

Effect of phosphate stress on leaf inclination in cereal and forage crops, and the importance of signalling molecules, ROS and NO

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Abstract

Phosphorus is a critical nutrient in plant development and reproduction, but levels of inorganic phosphate in soil are often insufficient for optimal crop growth. Rice plants have developed an adaptation to phosphate stress, by reducing leaf inclination through changes in lamina joint development. Currently, there is however, a lack of research on how phosphate stress affects leaf inclination in other important cereals and forage crops.

In this thesis, it was of interest to determine whether maize (*Zea mays L*), wheatgrass (*Triticum aestivum*), and Italian ryegrass (*Lolium multiflorum*) would exhibit reduced leaf inclination under insufficient phosphate. Furthermore, the possible involvement of hormone and phosphate status interactions to regulate leaf inclination was investigated. The involvement of signalling molecules, reactive oxygen species (ROS) and nitric oxide (NO), in phosphate stress-induced leaf inclination was also investigated.

The results obtained showed that *Lolium multiflorum* and *Triticum aestivum* displayed a decrease in leaf inclination under insufficient phosphate, while *Zea mays L* leaf inclination decreased, but this was non-significant. Transverse cross section analysis of the *Triticum aestivum* lamina joint demonstrated that there was no difference in abaxial and adaxial region width or cell layers under differing phosphate status.

Brassinosteroid had the strongest effect on leaf inclination, increasing leaf inclination in seedlings grown under both sufficient phosphate (+Pi) and insufficient (-Pi) conditions (2 fold increase under insufficient phosphate). Ethylene marginally increased leaf inclination in seedlings grown under either phosphate supply conditions, while auxin increased leaf inclination slightly in seedlings grown under -Pi conditions over that under +Pi conditions.

Increased production of ROS under phosphate stress was demonstrated. Qualitative assays showed increased production of superoxide and hydrogen peroxide in the marginal, adaxial side of the lamina joint of *Triticum aestivum*. No difference in lamina joint peroxidase activity was observed under either -Pi or +Pi conditions, however, increased lipid peroxidation activity was observed in the lamina joint of seedlings grown under -Pi conditions. Exogenous application of brassinosteroid reduced lipid peroxidation in seedlings grown under -Pi conditions. Finally, an NO donor, SNP, reduced leaf inclination in seedlings grown under +Pi conditions, although this decrease was non-significant.

Overall, these findings demonstrate that Italian ryegrass and wheatgrass have also developed a mechanism to respond to phosphate stress, by reducing leaf inclination. Increased ROS in the lamina joint of -Pi-grown seedlings strongly suggests that ROS could be an important signalling molecule in inducing this physiological response. At present, it remains unclear whether NO has a signalling role in phosphate induced leaf inclination.

Table of contents

Abstract	2
Acknowledgements	6
Abbreviations	7
List of Figures.....	8
1. Introduction and Literature Review.....	10
1.1. General Introduction	10
1.2. Phosphate stress and effect on leaf inclination.....	11
1.3. Liguleless genes in leaf inclination.....	12
1.4 Phosphate starvation response genes.....	12
1.5 Plant hormone interactions with leaf inclination	13
1.6 Hydrogen Peroxide and Nitric Oxide signalling	13
1.7 Objectives	15
1.8 Research questions and Hypothesis	16
2. Materials and Methods	17
2.1. Plant selection	17
2.2. Growth medium selection	19
2.3. Surface sterilisation of seeds	20
2.4. Growth Conditions (Environment)	20
2.5. Phosphate Starvation experiments	21
2.5.1 <i>Measurement of Leaf Inclination</i>	21
2.5.2 <i>Cross section analysis</i>	22
2.6. Plant hormone-nutrient interaction experiments (<i>Triticum aestivum</i>)	22
2.6.1 <i>Auxin experiments</i>	23
2.6.2. <i>Abscisic acid experiments</i>	23
2.6.3. <i>Cytokinin experiments</i>	23
2.6.4. <i>Brassinosteroid experiments</i>	23
2.6.5. <i>Ethylene experiments</i>	23
2.7. Reactive oxygen species (ROS) detection and quantification experiments	24
2.7.1 <i>Superoxide assay</i>	24

2.7.2. Hydrogen peroxide assay..	24
2.7.3. Peroxidase assay.....	24
2.7.4. Lipid Peroxidation assay....	25
2.7.5. Lipid peroxidation assay- Phosphate status and BR	26
2.8. Nitric Oxide donor experiments	26
2.8.1. NO donor SNP treatment to seedlings under differing phosphate status	26
2.8.2. cPTIO and SNP treatment to seedlings under differing phosphate status.....	26
2.8.3. cPTIO seed pre-treatment to seedlings under differing phosphate status	27
2.9. Data analysis	27
3. Results and Discussion	28
3.1 Phosphate Starvation induced leaf inclination	28
3.1.1. Italian Ryegrass.....	28
3.1.2. Sweet Corn (Maize)	30
3.1.3. Wheatgrass	31
3.2 Cross section of <i>Triticum aestivum</i> Lamina Joint.....	33
3.2.1. Transverse Lamina joint cross section under differing phosphate status	33
3.3 Interaction between Phosphate status and PGRs on leaf inclination	36
3.3.1. Control experiment (ddH ₂ O)	36
3.3.2. Auxin experiments (IAA)....	37
3.3.3. Absciscic Acid experiments (ABA).....	38
3.3.4. Cytokinin experiments (BA).....	39
3.3.5. Brassinosteroid experiments (BL application and inhibition).....	40
3.3.6. Ethylene experiments (ET application and inhibition)	42
3.4. Effect of phosphate status on ROS production (<i>Triticum aestivum</i>)	44
3.4.1 Superoxide assay (Qualitative).....	44
3.4.2. Hydrogen peroxide assay (Qualitative).....	45
3.4.3. Peroxidase assay (Quantitative)	46
3.4.4. Lipid peroxidation assay (Quantitative).....	47
3.4.5. Lipid peroxidation assay on seedlings grown with exogenous brassinolide ..	48
3.5. Effect of exogenous NO on leaf inclination (<i>Triticum aestivum</i>)	49
3.5.1 Effect of SNP treatment on leaf inclination under differing phosphate status.	49

3.5.2. <i>Effect of SNP and cPTIO treatment on leaf inclination under differing phosphate status</i>	50
3.5.3. <i>Effect of cPTIO seed pre-treatment on leaf inclination under differing phosphate status</i>	51
4. Final Conclusions and Future Directions	52
4.1 Key Findings	52
4.2 Limitations of Study	53
4.3 Future Research Suggestions.....	54
5. References	55
5.1. Supplementary Figures	60

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Abbreviations

cPTIO- 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
potassium salt

h- hours

MDA- Malondialdehyde

NO- Nitric oxide

PHR-Phosphate starvation responsive genes

PGR- Plant Growth Regulator

+Pi- Phosphate sufficient conditions

-Pi- Phosphate insufficient conditions

SNP- Sodium nitroprusside

TBARS- Thiobarbituric acid assay

List of Figures

Figure 1. Sweet corn (*Zea mays. L*) seeds.

Figure 2. Italian ryegrass (*Lolium multiflorum*) seeds.

Figure 3. Wheatgrass (*Triticum aestivum*) seeds.

Table 1. Half Strength MS media components.

Table 2. Reaction mixture for peroxidase assay

Table 3. Reaction mixture for lipid peroxidation assay

Figure 4. Visual representation of the effect of phosphate status on *Lolium multiflorum* leaf inclination.

Figure 5. The effect of phosphate status on leaf inclination of *Lolium multiflorum*.

Figure 6. Visual representation of the effect of phosphate status on *Zea mays L* leaf inclination.

Figure 7. The effect of phosphate status on leaf inclination of *Zea mays L*.

Figure 8. Visual representation of the effect of phosphate status on *Triticum aestivum* leaf inclination.

Figure 9. The effect of phosphate status on leaf inclination of *Triticum aestivum*.

Figure 10. Transverse cross section of *Triticum aestivum* lamina joint developed under insufficient phosphate.

Figure 11. Transverse cross section of *Triticum aestivum* lamina joint developed under sufficient phosphate.

Figure 12. Measurement of the width of m1 and m2 regions under -Pi and +Pi status

Figure 13. The number of cell layers in m1 region and sclerenchyma cell layers in m2 region under –Pi and +Pi status.

Figure 14. The effect of 48 h double distilled water incubation on leaf inclination of seedlings grown under differing phosphate status.

Figure 15. The effect of 48 h exogenous IAA incubation on leaf inclination of seedlings grown under differing phosphate status.

Figure 16. The effect of 48h exogenous ABA incubation on leaf inclination of seedlings grown under differing phosphate status.

Figure 17. The effect of 48 h exogenous BA incubation on leaf inclination of seedlings grown under differing phosphate status.

Figure 18. The effect of 48 h exogenous BL incubation on leaf inclination of seedlings grown under differing phosphate status.

Figure 19. The effect of 48 h UCZ incubation on leaf inclination of seedlings grown under differing phosphate status.

Figure 20. The effect of 48 h exogenous ET incubation on leaf inclination of seedlings grown under differing phosphate status.

Figure 21. The effect of 48 h AgNO₃ incubation on leaf inclination of seedlings grown under differing phosphate status.

Figure 22. Superoxide production in *Triticum aestivum* lamina joint under differing phosphate status.

Figure 23. Hydrogen peroxide production in *Triticum aestivum* lamina joint under differing phosphate status.

Figure 24. Peroxidase activity of lamina joint under differing phosphate status.

Figure 25. MDA content of lamina joint under differing phosphate status.

Figure 26. MDA content of lamina joint under differing phosphate status and exogenous BR.

Figure 27 The effect of SNP (200µM) treatment on leaf inclination under differing phosphate status.

Figure 28. The effect of SNP (150 µM) and cPTIO (80 µM) treatment on leaf inclination under differing phosphate status.

Figure 29. The effect of cPTIO (100 µM) seed pre-treatment on leaf inclination under differing phosphate status.

1. Introduction and Literature Review

1.1 General Introduction

Physiology of crop development is a rapidly growing area of science with the primary motivations to improve the resistance of various crops to both pesticides and herbicides, and also to maximise the amount of crop yield in an agricultural field. Cereal grain crops are the most important source of food worldwide, and are grown in quantities greater than any other crops, with wheat and rice making up over 50% of the cereal production worldwide (Food Science and Technology. 2008). Other important cereal crops such as maize are also an important source of food, where there is a rapidly growing demand of this crop, with 840 million megatons produced in 2010 (Lobell et al., 2013). Generally speaking, food production needs to double in the next 35 years, as of 2003, to meet future needs, and this has to be achieved despite the ongoing effects of climate change (Jones et al., 2003). Due to the impact of climate change, an overall loss of 10% in maize production in Africa and Latin America is projected up to the year 2055, which equates to a loss of 2 billion dollars a year (Jones et al., 2003). There clearly is an ongoing need to improve crop yield to meet demand in response to rapid human population growth. More locally, in New Zealand, forage crops such as ryegrass are an essential source of feed, where the two most important species are annual ryegrass (*Lolium multiflorum* Lam) and perennial ryegrass, which have a coverage of at least 7 million hectares over New Zealand (Trethewey et al., 2016; Bajaj et al., 2010). The success of the dairy farming industry in New Zealand is fairly dependent on the quality and quantity of feed such as ryegrass, so yield needs to be improved to meet a growing demand for animal feed in dairy farming.

Maize yield in the US has increased dramatically by about eight-fold in the last 80 years, due largely to the adaptation of hybrid plants to increasingly higher maize plant densities (Tian et al., 2011). Increases in yield are also attributable to greater tolerance to stress, resulting in yield improvement by 2.5% per year from the 1950s to the late 1980s (Tollenaar and Wu. 1999). In the last 50 years, maize plant density has increased by about 1000 plants ha/yr in the US Corn Belt (Duvick. 2005).

1.2 Phosphate stress and effect on leaf inclination

Plants naturally spread their leaves to maximise capturing of incoming sunlight, but those that can hold their leaves more upright would decrease mutual shading, and can therefore be grown at much higher densities (Mach. 2018). While many crops have developed more upright leaves due to adaptation and also in part by selective breeding, little is currently known on what role nutrients have on leaf angle. The essential nutrient, phosphorus, is required for plant development and reproduction, and makes up a major component of fertilisers that are used in agriculture (Lopez-Arredondo et al., 2014). The levels of inorganic phosphate (Pi) in soil are often insufficient for optimal growth and crop productivity. Some microorganisms can enhance plant Pi uptake, while others compete with roots for Pi uptake (Lopez-Arredondo et al., 2014). In practice, excessive fertilisers are applied in crop fields, leading to the eutrophication of waterways and formation of toxic algal blooms (Lopez-Arredondo et al., 2014). Clearly, there is a need to meet food demands in a much more sustainable way while also not having detrimental impacts on other important ecosystems (Cakmak. 2002),

While there is a need for improvement in terms of phosphate acquisition efficiency and phosphate utilisation efficiency in plants, there has been evidence that plants have been able to adapt to low-Pi availability (Lopez-Arredondo et al., 2014). A common observation of plants which are grown with insufficient Pi, is an erect-leaf phenotype. This area of research is very recent, and has been largely investigated in rice, where Ruan et al (2018) examined the effects of Pi on leaf angle in rice (*Oryza sativa*). The lamina joint, which links the leaf blade to the leaf sheath, is responsible for the leaf angle, where elongation of cells in this joint results in a larger leaf angle (Mach. 2018). In a recent study (Ruan et al., 2018), it was found that when rice plants were grown under insufficient Pi, the cells on the abaxial and adaxial side of the lamina joint were shorter, resulting in a smaller leaf angle. The regulation of leaf inclination is controlled by SPX proteins, SPX1 and SPX2, which interact and inhibit the transcription factor, RLI1 (Regulator of Leaf Inclination1) (Ruan et al., 2018). Furthermore, they found that RLI1 activates BU1 and BC1 transcription factors, which increase leaf angle, by the promotion of cell elongation in the lamina joint. So under insufficient Pi, this induced the expression of SPX1 and SPX2, which repressed RLI1, BU1 and BC1 (Ruan et al., 2018).

1.3 Liguleless genes in leaf inclination

Maize leaves are made up of a blade which is separated from the leaf sheath by a ligular region, which consists of an auricle and a ligule (Sylvester et al., 1990). Similarly, in rice, the lamina joint links the leaf sheath and leaf blade (Hoshikawa. 1989).

Both the auricle and ligule influence leaf angle, where Sylvester et al (1990) found that mutant plants expressing the recessive *liguleless-1* (*lg1*) allele displayed loss of normal ligules and auricles. The *liguleless-1* gene clearly affects maize leaf development, and the recessive mutation prevents the formation of ligules and auricles (Lee et al., 2007). The *lg1+* wild type gene seems to act in a tissue specific manner at an early stage of ligule and auricle initiation (Becraft et al., 1990). Further evidence for *liguleless-1*'s role in the formation of ligule and auricle is supported by the presence of a nuclear-localized protein, encoded by the *lg1* gene (Moreno et al., 1997). More recently, Tian et al (2011) carried out a genome-wide association study of leaf architecture in maize. The main findings of this study were that variations in *liguleless* genes contribute to more upright leaves. This gives additional evidence for the importance for *lg1* genes in determining leaf angles in maize. Interestingly, a number of liguleless genes also function during leaf initiation or lateral branching (Johnston et al., 2014). Various other *liguleless* mutants, such as *Oslg1* in rice, also display the loss of ligules and auricles, while an *lg1* homolog in wheat (*TaSPL8*) also has a conserved role in regulation of leaf inclination (Liu et al., 2019)

The auricle effectively acts as a hinge, which allows the leaf blade to project out at an angle from the stem, and it was tested whether the auricle affects leaf angle or not (Kong et al., 2017). There was a strong positive correlation between auricle angle and leaf angle, and also between auricle size and leaf angle (Kong et al., 2017). These results are consistent with those of Moreno et al (1997), showing that reduced auricle development resulted in small leaf angles, as seen in *lg1* and *lg2* mutants. The determinants of final auricle size and leaf angle were based on the number of cells, and size of cells, generated by cell division and cell elongation respectively (Kong et al., 2017). Maize lines with non-upright leaves showed higher cell division rates and elongated more than those with upright leaves, and so had a wider auricle angle and leaf angle (Kong et al., 2017).

1.4 Phosphate starvation response genes

Of particular importance to the persistence of cereal plants are genes that are expressed in response to stress. Those of interest are phosphate starvation response (PHR) genes. Many of these genes are found in gramineous plants such as maize and rice, and based on gene expression analysis, many PHR genes exhibited upregulated expression in response to low phosphate stress. This suggests that certain PHR genes may be important in response to phosphate stress (Xu et al., 2018). Yu et al (2019) were able to isolate a gene, *ZmAPRG*

(uncharacterised) that enhances acid phosphatase activity (APA) and the concentration of phosphate in maize leaves during Pi starvation. Acid phosphatase is important in the uptake and use of Pi during maize growth, and APA activity and Pi concentration increased significantly when *ZmAPRG* was overexpressed (Yu et al., 2019). *ZmAPRG* is clearly an important gene in the response to phosphate starvation.

1.5 Plant hormone interactions with leaf inclination

Along with the strong genetic basis known to regulate leaf inclination in many cereal crops, many important plant growth regulators (PGRs) such as auxin and brassinosteroids (BR) are also crucial in leaf angle determination. Various BR -deficient mutants such as dwarf, dwarf1, and d61-7 (BR signalling mutants) showed erect leaves, while BR overexpression resulted in large leaf inclination (Jang and Li. 2017). The role auxin plays in controlling leaf inclination seems to require crosstalk with brassinosteroid, which can probably explain why auxin and BR signalling share many genes (Zhang et al., 2015). It has been demonstrated that auxin response factors (ARF) are related to regulation of leaf inclination, as a loss of function ARF mutant displayed a reduction in leaf angle of flag leaves relative to the wild type (Zhang et al., 2015). *OsARF19* could control expression of *OsBR11*, and so regulate BR signalling, showing the interrelationship between auxin and brassinosteroid signalling (Zhang et al., 2015). Evidence of auxin and brassinosteroid regulation of leaf inclination has also been found in wheat. It was found that the gene, *TaSPL8*, was responsible for activation of genes related to auxin signalling and BR biosynthesis. When *TaSPL8* was knocked out, the mutants displayed erect leaves (Liu et al., 2019).

Another PGR, ethylene, may also contribute to plant hormone crosstalk in leaf inclination. While there are no studies to date suggesting that it regulates leaf inclination, it is involved in crosstalk with brassinosteroid in a number of different plant development responses. For example, a bioactive BR, EBR, triggered synthesis of ethylene, which was able to mediate stomatal closure in *Arabidopsis thaliana* in conjunction with Gα protein (Shi et al., 2015). The intimacy between ethylene and brassinosteroid suggests that ethylene has a signalling function downstream of BR in the regulation of leaf inclination.

1.6 Reactive oxygen species and nitric oxide signalling

As plants undergo oxidative stress from macronutrient deficiencies, this causes the plant to produce and quickly build up free radicals, or reactive oxygen species (ROS). One of the ROS that has received the most attention, H₂O₂, is generated from redox reactions that occur in

the chloroplasts or mitochondria, however the main source of H_2O_2 is an NADPH-dependent oxidase (Hossain et al., 2015). Counteracting the endogenous increase in ROS is an antioxidant response to keep the generation of ROS under control, where antioxidative enzymes such as superoxide dismutase, catalase and peroxidase are important in this response (Tewari et al., 2004). It was found that under phosphorus deficiency, there was a significant increase in activity of catalase, however, this deficiency did not affect the concentration of ROS, hydrogen peroxide (H_2O_2) in the leaves (Tewari et al., 2004). In contrast, bean plants under sustained phosphate starvation had increased lipid peroxidation and hydrogen peroxide concentration in their root tissues (Juszczuk et al., 2001). It is possible that antioxidant enzymes limit the concentration of H_2O_2 in plant tissues, as it was found in these maize plants that there was a significant increase in superoxide dismutase (SOD) activity (Tewari et al., 2004).

While hydrogen peroxide can initiate an antioxidant response to oxidative damage, it also acts as a signalling molecule to mediate processes such as cell division, cell differentiation, and cell migration (Veal et al., 2011). Hydrogen peroxide can act as a messenger to carry a redox signal from the site of generation to the target site, where H_2O_2 modulates the activity of various transcription factors in bacteria, in lower eukaryotes, and in mammal cells (Sies, 2017). Several genes encoding transcription factors were found to be upregulated in response to H_2O_2 . It seems that H_2O_2 plays an important signalling role at just about every stage of plant development, and also under numerous abiotic stresses (Niu and Liao, 2016). There is also considerable evidence that both hydrogen peroxide and nitric oxide are important signalling molecules in plants (Neill et al., 2001). For example, H_2O_2 was found to induce the gene expression of four defence-related genes in *Arabidopsis thaliana* (Desikan et al., 2000). A MAPK cascade (protein phosphorylation cascade) is activated by hydrogen peroxide, which upon activation allows it to phosphorylate transcription factors, activating them (Neil et al., 2001). Similarly, NO has been shown to be an extremely versatile signalling molecule, which is involved in abiotic stress responses, and processes such as seed germination, flowering, ripening and senescence, and mediation of stomatal closure (Niu and Liao, 2016).

Nitric oxide has also been suggested to have roles in the control of root growth, and also as a maturation and senescence factor, while the generation of NO has been detected under the same conditions of H_2O_2 generation (Neil et al., 2001). Nitric oxide is typically produced by nitric oxide synthase or nitric reductase (Qiao et al., 2015).

Often, the presence of both NO and H_2O_2 signals is important in stress responses, and both signals act together to activate a MAPK cascade. There is a significant ongoing debate in regard to whether H_2O_2 operates upstream of NO or not. In a heat shock pathway in *Arabidopsis*, hydrogen peroxide operated upstream of nitric oxide (Wang et al., 2014), and in response to abscisic acid, H_2O_2 regulates NO during the process of stomatal closure (Bright et al., 2006). In contrast, NO is responsible for accumulation of H_2O_2 following herbivory attacks or Tobacco Mosaic Virus infection (Si et al., 2017).

Hydrogen peroxide is not the only ROS important in the signalling response to biotic and abiotic stress, as superoxide has also been demonstrated to have a signalling role in response to plant-pathogen interactions (Cvetkovska et al., 2013). Furthermore, superoxide serves as

a signalling molecule in plant cell division, promoting cell cycle in *Capsicum annum* (Lee et al., 2017).

1.7 Objectives

The effects of phosphate deficiency on leaf angle in rice have been investigated, demonstrating that plants deficient in phosphate show an erect leaf angle phenotype (Ruan et al., 2018). However, there are no studies to date on the effect of phosphate starvation on leaf angle in other important cereal crops, such as maize and wheat, despite the significant amount of literature available on the importance of the *liguleless-1* gene and other loci important in influencing leaf angles of maize and rice plants. Furthermore, there are gaps in the knowledge of the importance of hydrogen peroxide and nitric oxide in signalling and regulating stress-related responses of plants. In particular, it is unclear whether hydrogen peroxide and nitric oxide have a signalling role in phosphate stress-related leaf inclination.

In response to phosphate starvation, there is expression of various PHR genes in maize and other cereal plants. As these genes are crucial for the survival of plants during abiotic and biotic stress, these genes may have important interactions with signalling molecules such as hydrogen peroxide and nitric oxide. It is well recognised that brassinosteroids are important in regulating leaf angle. For example, wheat gene, *TaSPL8*, affects leaf inclination by activating genes important in BR and auxin pathways (Liu et al., 2019), and overexpression of BR biosynthesis genes has been found to result in larger leaf inclination (Jang and Li. 2017). Despite this knowledge, the way in which phosphate status interacts with hormones such as brassinosteroids is unknown. Answering this question would allow us to understand whether crosstalk between hormone and nutrient can influence leaf inclination, and improve current strategies on maximising crop yield.

The erect leaf phenotype seems to be an important adaptation to maximise efficiency of photosynthesis in response to phosphate stress. There is clearly a requirement for ongoing research into understanding the signalling responses that induce a change in leaf angle. This will be invaluable in improving productivity and yield of many important cereal crops. For example, a consistent phenotype among maize plants in terms of leaf angle, could allow many more plants to be arranged in a plot, and allow maximal light capture (Torres et al., 2011).

There seems to be a positive association between phosphate starvation and hydrogen peroxide concentration. An example in rice plants showed that the expression of arsenate reductase gene was positively correlated with root H_2O_2 accumulation, increasing in response to phosphate starvation (Wang et al., 2008). While hydrogen peroxide and other ROS initiate an antioxidant response, they may also serve an important signalling role in the determination of leaf angle in cereal and forage crops.

1.8 Research question and Hypothesis

The aim of this project was to investigate the importance of phosphate starvation in regulation of leaf inclination of maize, Italian ryegrass, and wheatgrass. An investigation into how phosphate status might interact with hormones to regulate leaf inclination was conducted, and finally, ROS and nitric oxide were examined for their potential as important signalling molecules in influencing leaf inclination. Specifically, it was of interest to determine whether an endogenous increase in ROS and / or nitric oxide was associated with a decrease in the leaf inclination of cereal and forage crops.

Hypothesis 1: Depriving cereal and forage plants (sweet corn, Italian ryegrass, wheatgrass) of phosphate would result in a decrease of leaf inclination compared to control plants.

Hypothesis 2: Various plant growth regulators (brassinosteroids, ethylene, and auxins) interact with phosphate status in the regulation of leaf inclination.

Hypothesis 3: ROS are important in the negative regulation of leaf inclination

Hypothesis 4: Nitric oxide is important in the negative regulation of leaf inclination

2. Materials and Methods

2.1 Plant material

Seedlings were raised from germinating the following types of seeds (Figures 1-3).

Sweet corn seeds (*Zea mays* L.) were obtained from Mr Fothergill's Seeds NZ (Christchurch, NZ)

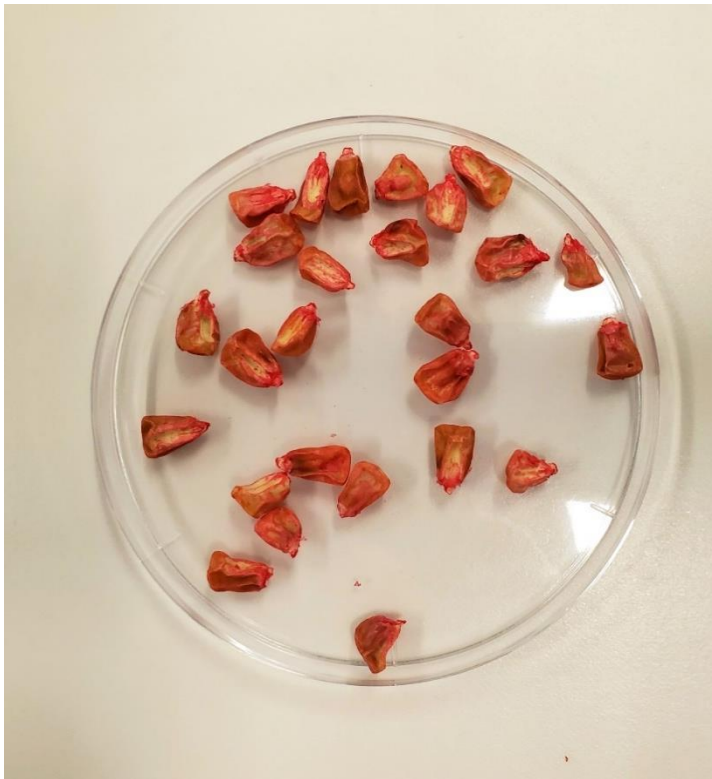


Figure 1. Sweet corn (*Zea mays*. L) seeds. Photo taken by Nathan Hulston (2020).

Italian ryegrass seeds (*Lolium multiflorum*) were obtained from Kings Seeds (Katikati, NZ)



Figure 2. Italian ryegrass (*Lolium multiflorum*) seeds. Photo taken by Nathan Hulston (2020).

Wheatgrass seeds (*Triticum aestivum*) were obtained from Kings Seeds (Katikati, NZ)



Figure 3. Wheatgrass (*Triticum aestivum*) seeds. Photo taken by Nathan Hulston (2020).

2.2 Growth Medium Selection

Murashige and Skoog (MS) basal medium was chosen as the growth medium in this study. The stock solutions (1 L) of half strength Murashige and Skoog (MS) medium were made up as follows (Murashige and Skoog. 1962).

Components	X	Volume prepared	Chemical name	Formula	Amount
Major salt(g)	10x	1000ml	Ammonium nitrate	NH ₄ NO ₃	8.25 g
			Potassium nitrate	KNO ₃	9.5 g
			Calcium chloride 2-hydrate	CaCl ₂ .2H ₂ O	2.2 g
			Magnesium sulphate anhydrous	MgSO ₄	0.903 g
			Potassium dihydrogen orthophosphate	KH ₂ PO ₄	0.85 g
Minor Salt(g)	100x	1000ml	Potassium iodide	KI	0.0415 g
			Boric acid	H ₃ PO ₃	0.310 g
			Manganese sulphate	MnSO ₄ .4H ₂ O	1.115 g
			Sodium molybdate	Na ₂ MoO ₄ .H ₂ O	0.0125 g
			Cupric Sulphate	CuSO ₄ .5H ₂ O	0.00125 g
			Cobalt chloride	CoCl ₂ .6H ₂ O	0.00125 g
			Zinc sulfate heptahydrate	ZnSO ₄ .7H ₂ O	0.430 g
FeSo4(g)	100x	200ml	Iron(III) sulphate 7-hydrate	FeSO ₄ .7H ₂ O	0.556 g
Na2EDTA(g)	100x	200ml	Disodium salt of ethylenediaminetetraacetic acid	Na ₂ EDTA	0.746 g
Vitamins(g)	100x	200ml	Myo-inositol	Myo-inositol	2 g
			Glycine	Glycine	0.04 g
			Nicotinic acid	Nicotinic acid	0.01 g

			Pyridoxine HCl	Pyridoxine HCl	0.01 g
			Thiamin HCl	Thiamin HCl	0.002 g

Table 1. Half Strength MS media components

As per the requirements for phosphate sufficient (+Pi) and phosphate deficient (-Pi) treatments, two MS major salt stocks were made up. The +Pi stock contained all the substituents described in the table above, while in the -Pi stock, potassium dihydrogen orthophosphate was substituted for an equivalent concentration of potassium chloride (0.465g).

The following amounts were taken from the stock solutions for 400ml of MS medium:

1. Major salt: 40 ml
2. Minor Salt: 4 ml
3. FeEDTA: 4 ml
4. Vitamins: 6 ml

The medium was supplemented with 1.5% (w/v) sucrose, and the pH of the medium was adjusted to 5.8 using HCl and NaOH. Following this adjustment, 1% (w/v) agar was added before the medium was autoclaved.

Approximately 40ml of the medium was poured into each 250 ml autoclaved polycarbonate growth container.

The media was left to solidify for 30 minutes.

2.3 Surface Sterilization of Seeds

During the last 20 minutes of agar solidification, the respective seeds (sweet corn, Italian ryegrass, and wheatgrass) were sterilised in 15% (v/v) bleach with 5% (w/v) sodium hypochlorite as the active ingredient for 20 minutes, before being rinsed 6 to 7 times in sterile water. Sterile forceps were used to place a seed on the surface of the medium in each growth container.

2.4 Growth conditions (environment)

All seedlings were developed under *in vitro* conditions. In all the preliminary experiments and the early part of the project, growth containers with the seedlings chosen for this project were placed in a plant growth incubator kept at a temperature of 23°C and with constant lighting. The positioning of lights on the side of the incubator had an influence on the directionality of plant growth. Rather than growing upright, seedlings displayed more

curvature in their growth. The control and the seedlings in all the treatments were subject to the same photoperiod and temperature.

After six months of using the above plant growth incubator, seedlings were developed in a plant growth room with a constant temperature of 23°C, and a photoperiod of 24 hours. The L.E.D lights in the growth room were positioned vertically above the growth jars, and the light intensity at the top of the jars was $12.1 \mu\text{mol m}^{-2} \text{s}^{-1}$.

2.5 Phosphate starvation experiments

A selection of healthy looking seeds was made before they were germinated on half strength MS media. Potassium chloride was used to substitute for Pi under Pi deficient conditions, as this substitute did not appear to interfere with the other salts in the major salt stock solution.

Italian ryegrass

After surface sterilisation, 4 seeds were placed 2 cm apart on the surface of $\frac{1}{2}$ strength MS (+Pi) medium in a 250 ml growth container (5 replicate jars). In comparison, there were replicate jars with seeds placed on $\frac{1}{2}$ strength MS (-Pi) medium. Later on in the project (about 5 months), there were only 3 seeds in each container in order to improve light interception of each seedling.

Sweet corn

One surface-sterilised seed was placed in an Agee glass jar (3 replicate jars) containing approximately 50 ml of $\frac{1}{2}$ strength MS (+Pi) or (-Pi) medium. Petri dish lids were used to cover the top of the Agee jars, and were sealed on using gladwrap.

Wheatgrass

Three surface-sterilised seeds were placed 3 cm apart on the surface of the medium in a 250 ml growth container. There were 5 replicate containers with $\frac{1}{2}$ strength MS (+Pi) medium and 5 replicate containers with $\frac{1}{2}$ strength MS (-Pi) medium.

2.5.1 Measurement of leaf inclination

Leaf inclination was measured as the angle between the first and secondary leaf based on a protocol adapted from Yoshida et al (1969). It was deemed unnecessary to measure leaf inclination against a vertical board as seedling leaves did not droop much, due to the agar providing great structural support. This made leaf inclination measurements much more efficient. Leaf inclination measurements were taken following development of secondary leaf, and the lamina joint on the primary leaf. For hormone-nutrient interaction experiments, photos of lamina joints were captured against a plain white background, and a distribution of ImageJ software, Fiji, was utilised to measure leaf inclination, by use of the angle tool. (Available from <https://imagej.net/Downloads>).

2.5.2 Cross section analysis

Cross sections of lamina joints from phosphate-sufficient and -deficient plants were produced following an adapted protocol from Ruan et al (2018).

After the +Pi and -Pi treatments, excised lamina joints were fixed in FAA solution (3% formalin, 5% glacial acetic acid, 30.5% ethanol, and 61.5% distilled water), under vacuum pressure over approximately 12 h.

Post fixation, the lamina joints were processed through a series of washes to remove FAA solution within plant cells (see the protocol below):

1. 95% ethanol x1
2. 80% ethanol x1 ≤ 30 minutes
3. 50% ethanol x2
4. dH₂O x2

Following washes, the plant materials were embedded in 3% (w/v) agar (agar was poured at a temperature of 40°C).

Transverse cross sections (30 µm) were then sectioned using a Vibratome 1000 Plus (The Vibratome Company, St Louis, MO, USA). Speed of the Vibratome was set to 8 (scale of 10), while the amplitude was set to 5. A sectioning blade was set to an angle of 40°.

Sections were then observed and photographed under a Leica TCS SP5 confocal microscope with a 63x 1mm and 20x 1mm objective lens. Wavelength range used was 415nm-485nm. All cell measurements were made with ImageJ software.

2.6 Plant hormone-nutrient interaction experiments (*Triticum aestivum*)

Several plant growth regulators (PGRs) were utilised to investigate how they might interact with phosphate status to regulate leaf inclination. The chosen PGRs were, epibrassinolide (EBI), indole-3 acetic acid (IAA), 6-benzylaminopurine (BA), abscisic acid (ABA), and ethylene. Brassinolide inhibitor, uniconazole (UCZ), and an ethylene inhibitor, silver nitrate, were also used singly (not in combination with other PGRs).

A number of different interaction experiments were designed. We decided upon carrying out a micro quantitative bioassay, adapted from Wada et al (1984). 100ml Stock solutions (100 mL each) of PGR and phosphate mixtures were prepared, using double distilled water (ddH₂O). No phosphate was added to the stock solutions for seedling growth under insufficient phosphate (-Pi), while dihydrogen ortho phosphate (the same concentration as used in 1/2x MS medium) was added to the stock solutions for seedling growth under

sufficient phosphate conditions (+Pi). Only phosphate was used in combination with PGRs, as it is not known if other components in the MS medium might have an effect on leaf inclination.

For each treatment, 5 seedlings were excised, leaving 1 cm of primary leaf, 1 cm of secondary leaf, and 1 cm of stem. Prior to exposing the excised segments to PGR treatment, they were incubated in double distilled water for 24 h (in the dark). This was to ensure that any increase or decrease in leaf inclination would be due to PGR-phosphate treatment, and not due to a gravitropic effect (explained in section 3.5.1).

The excised stem segments were then photographed against a plain white background, and leaf inclination was measured prior to hormone treatment. The segments were then placed in a Petri dish containing 20 mL of a nutrient-hormone mixture, covered with aluminium foil, and incubated in the dark at 25°C for 48 h. Following incubation, the excised segments were removed from solution, and photographed again using the same background. Any change in leaf inclination was measured with ImageJ software.

2.6.1 Auxin experiments

Indole-3-acetic acid (IAA) was applied to –Pi and +Pi stock solutions, to reach a final concentration of 28 µM in each solution. The pH of both treatments was adjusted to 5.8.

2.6.2 Absciscic acid experiments

Absciscic acid (ABA) was applied exogenously to –Pi and + Pi stock solutions to reach a final concentration of 19 µM. The pH of both solutions was adjusted to 5.8.

2.6.3 Cytokinin experiments

6-Benzylaminopurine (BA) was applied exogenously to –Pi and +Pi stock solution to reach a final concentration of 22 µM. The solutions for both treatments were adjusted to 5.8.

2.6.4 Brassinosteroid experiments

An active form of BR, epibrassinolide (EBL) was added to –Pi and +Pi stock solutions, to reach a final concentration of 8 µM in each solution. The pH of solutions was adjusted to 5.8.

A brassinosteroid inhibitor, uniconazole (UCZ), was applied to –Pi and +Pi stock solutions, to reach a final concentration of 70 µM in each solution. The pH of both solutions was adjusted to 5.8.

2.6.5 Ethylene experiments

Ethylene precursor, Ethephon, a solution that can release ethylene, was added to –Pi and +Pi stock solutions to reach a final concentration of 150µM. The pH of the solutions was adjusted to 4.

An inhibitor of ethylene action, silver nitrate (AgNO₃), was applied to –Pi and +Pi stock solutions to reach a final concentration of 180 µM. The pH of the solutions was adjusted to 5.6.

2.7. Reactive oxygen species (ROS) detection and quantification experiments

2.7.1 Superoxide assay (Qualitative)

The presence of superoxide was detected using nitro blue tetrazolium(NBT) staining, based on a protocol adapted from Kumar et al (2014).

Seedlings were grown for 14 days under +Pi and -Pi conditions before they were excised (segments containing 1 cm of primary leaf, 1 cm of leaf sheath, and 1 cm of secondary leaf) and placed into Falcon tubes containing 4 ml of 0.5 mM NBT. The Falcon tubes were then placed inside a vacuum desiccator, and were vacuum infiltrated for 2 h under half strength vacuum pressure (under low light conditions). Following infiltration, excised segments were removed from NBT solution, and placed in Falcon tubes containing of 95% ethanol.

Excised segments were then bleached in a water bath at 90°C for 2.5 h in order to remove chlorophyll. Bleached segments were then photographed against a plain white background, and observed under a stereo microscope.

2.7.2. Hydrogen peroxide assay (Qualitative)

Hydrogen peroxide was detected using a 3,3'-diaminobenzidine (DAB) staining assay, based on a protocol adapted from Kumar et al (2014).

Seedlings were grown for 14 days under +Pi and -Pi conditions before they were excised and placed into Falcon tubes containing 4 ml of 4 mM DAB (adjusted to pH 3.8, as used in Kumar et al., 2014). The Falcon tubes were then placed inside a vacuum desiccator, and were vacuum infiltrated for 2 h under half strength vacuum pressure (under low light conditions). Following infiltration, excised segments were removed from DAB solution, and placed in Falcon tubes containing 3 mL of 95% ethanol.

Excised segments were then bleached at 90°C for 2.5 h in order to remove chlorophyll. Bleached segments were photographed against a plain white background, before closer observation under a stereo microscope.

2.7.3. Peroxidase assay (Quantitative)

Guaiacol peroxidase activity was measured by the oxidation of electron donor guaiacol at the expense of H₂O₂ (Sharma et al., 2012).

Seedlings grown under +Pi and -Pi conditions were collected, following development of secondary leaf (14 days from time of sowing). The peroxidase assay performed was adapted from Baque et al (2010). Lamina joint sections (4 mm long; 2 per replicate from same jar) were excised from the seedlings, and each segment consisted of the leaf sheath, lamina joint, and leaf blade. The segments were then homogenised with a mortar and pestle on ice in KPO₄ buffer (50 mM, pH 6.9) to a total volume of 1.2 mL (4 x 0.3mL), before being transferred into an Eppendorf tube. The homogenate was then centrifuged at 4°C for 5 minutes at 10,000rpm.

After centrifugation, the supernatant was transferred to another Eppendorf tube, before 50 μ L of the supernatant (enzyme extract) was added to a reaction mixture (as shown in Table 2). Absorbance (Ab) of the reaction mixture was measured at 470 nm after 5 min.

Enzyme controls were also performed in the enzyme assays. Enzyme extract (100 μ L) from each relevant treatment was removed and boiled at 100°C for 5 min. The same volume (50 μ L) of boiled enzyme was used in a reaction mixture, instead of the unboiled supernatant (Table 2).

Table 2: Reaction mixture for peroxidase assay

Solution	Treatment	Enzyme control	Blank
KPO ₄ Buffer	943 μ L	943 μ L	993 μ L
H ₂ O ₂ (10%)	5 μ L	5 μ L	5 μ L
Guaiacol	2 μ L	2 μ L	2 μ L
Enzyme	50 μ L	50 μ L(boiled)	-

Total peroxidase activity was expressed as Ab unit/min/lamina joint.

2.7.4. Lipid Peroxidation assay (Quantitative)

Lipid peroxidation was detected by addition of thiobarbituric acid, which reacts with lipid peroxidation end product, malondialdehyde, giving a red colour (Hodges et al., 1999).

Seedlings grown under +Pi and -Pi conditions were collected, following development of secondary leaf (14 days from time of sowing). A Lipid peroxidation-thiobarbituric acid reactive substances (TBARS) assay was carried out, adapted from Hodges et al (1999). Lamina joint segments (4 mm long; 4 per replicate) were excised from seedlings, and each segment consisted of the leaf sheath, lamina joint, and leaf blade.

The segments were then homogenised with mortar and pestle on ice in 1.2 mL of 0.1% (w/v) trichloroacetic acid (TCA) before being transferred into an Eppendorf tube. The homogenate was then centrifuged at 4°C for 5 min at 10,000 rpm. After centrifugation, the supernatant was transferred into a new Eppendorf tube. An aliquot (0.5 mL) of the supernatant was added to a reaction mixture in a glass tube (see Table 3).

The reaction mixture was then incubated in an 85°C water bath for 25 min before being transferred into an Eppendorf tube and cooled on ice. Once cooled, the reaction mixture was centrifuged once again at 4°C for 5 min at 10,000 rpm. The absorbance was read at 532 nm and 600 nm which was subtracted from 532 nm reading to correct for nonspecific turbidity.

Table 3. Reaction mixture for lipid peroxidation assay

Solution	Treatment	Control
20% TCA and 0.5% thiobarbituric acid(TBA)	1000µL	-
20% TCA	-	1000µL
Enzyme	500µL	500µL

The molar extinction coefficient, $\epsilon=155\text{mM}^{-1}$ was used to calculate the concentration of the malondialdehyde (MDA) –TBA complex, which was expressed as nM MDA/lamina joint.

2.7.5. Lipid peroxidation assay- Phosphate status and BR

The concentration of the MDA-TBA complex was measured using the same protocol described above. Seedlings were grown for 14 days under +Pi and -Pi conditions , in the presence of 1.6 µM epibrassinolide.

2.8. Nitric Oxide donor experiments

2.8.1. NO donor SNP treatment under differing phosphate status

Phosphate deficient and sufficient half strength MS-media were prepared as per protocol described above. Seeds were surface sterilised using 15% bleach for 20 min.

Prior to sowing of seed, a sterile Eppendorf lid was placed on the agar surface in the centre of a growth jar. Sodium nitroprusside (hereafter referred as SNP; 12 µg/µL) was pipetted into the lid (200 µL). Surface sterilised seeds were then placed around the perimeter of the jar. For the control, Eppendorf lids were filled with 200 µL of sterile distilled water.

Following 14 days of growth, seedlings were removed from the jars, photographed against a plain white background, and leaf angle was measured using ImageJ, using the same protocol as in section 2.5.1.

2.8.2. NO scavenger cPTIO and SNP treatment under differing phosphate status

Phosphate deficient and sufficient half strength MS-media was prepared as per protocol described above. Seeds were surface sterilised using 15% bleach for 20 minutes.

Prior to sowing of seed, a sterile Eppendorf lid was placed on the agar surface in the centre of the growth jar. A solution containing 9 µg/µL SNP, and 5.2 µg/µL cPTIO was pipetted into the lid (200 µL). Seeds were then placed around the perimeter of the jar. For the control, Eppendorf lids were filled with 200 µl of sterile distilled water.

Following 14 days of growth, seedlings were removed from jars, photographed against a plain white background, and leaf angle was measured using ImageJ, using the same protocol described earlier.

2.8.3. cPTIO seed pre-treatment to seedlings grown under differing phosphate status

Half strength MS-media was prepared as per protocol described above. Seeds were surface sterilised using 15% bleach for 20 minutes. Following sterilisation, seeds were processed through a pre-treatment protocol, adjusted from Phang et al (2011), where seeds were soaked in sterile 100 μ M cPTIO solution for 3 h. For the control, a separate batch of seeds were soaked in sterile distilled water for 3 h. Seeds were then sown on phosphate sufficient or phosphate deficient MS-media.

After 14 days following sowing, seedlings were removed from growth jars, photographed against a plain white background, and leaf angle was measured with ImageJ.

2.9. Data analysis

All leaf inclination data were organised on Microsoft Excel (2013), before analyses were performed using the Prism package of GraphPad. One-tailed student t-tests were performed for phosphate stress experiments, and lipid peroxidation (based on phosphate status). Two-tailed student t-tests were performed for transverse section analysis (n=3). Repeated Measure two way ANOVAs were utilised to analyse hormone-phosphate interactions. Multiple comparisons were performed using Sidaks's test (n=4). One way ANOVA was carried out for peroxidase activity under differing phosphate status. Multiple comparisons were performed using Tukey's test (n=4). Standard 2 way ANOVAs were carried out for phosphate and nitric oxide interaction experiments, with multiple comparisons performed using Tukey's test (n=5).

Significant differences between treatments were established with $P < 0.05$.

3. Results and Discussion

3.1 Phosphate Starvation induced leaf inclination

3.1.1. Italian Ryegrass

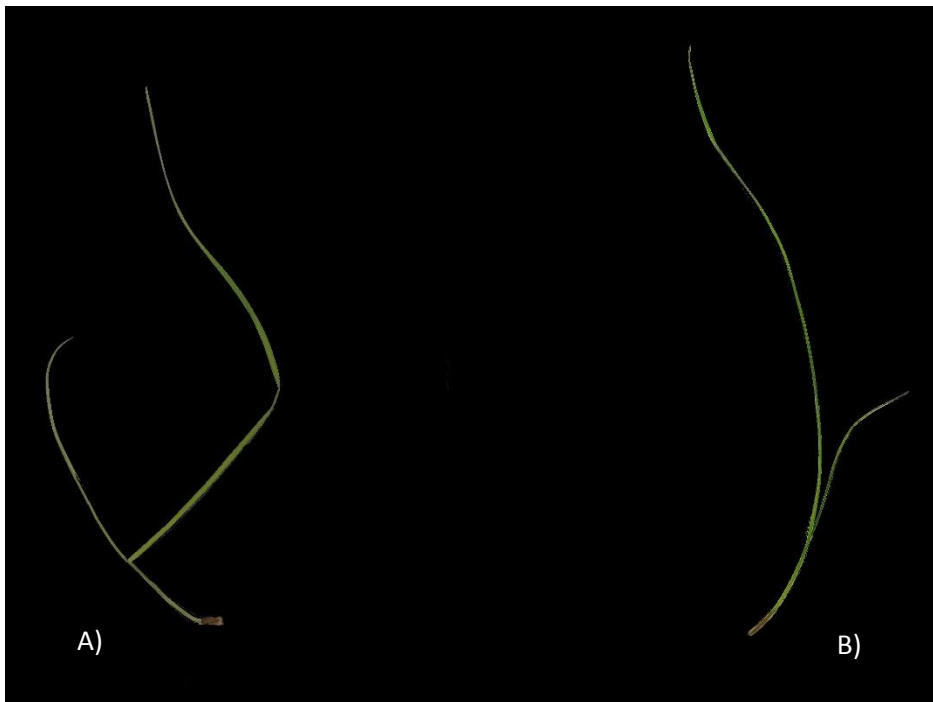


Figure 4. Visual representation of the effect of phosphate status on *Lolium multiflorum* leaf inclination. A) 14 day old seedling grown under sufficient phosphate. B) 14 day old seedling grown under insufficient phosphate.

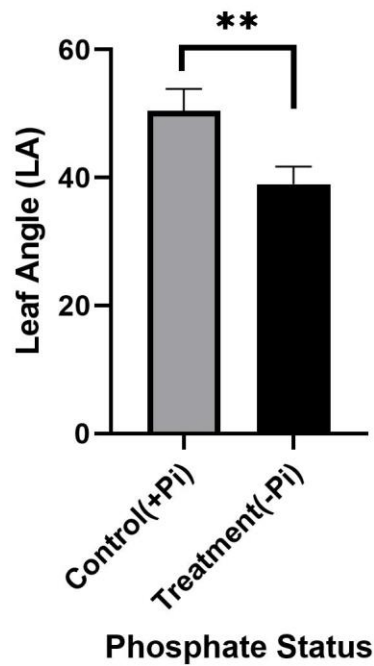


Figure 5. The effect of phosphate status on leaf inclination of *Lolium multiflorum*. Values indicated are mean leaf angle + SEM ($n=19$). Asterisks indicate the degree of significant difference.

Consistent with the findings of a previous study (Ruan et al., 2018), starving ryegrass seedlings of inorganic phosphate resulted in a reduced primary leaf angle (Figures 4 and 5). The average leaf angle of the primary leaf under sufficient phosphate was 50°, while under insufficient phosphate the average leaf angle was 39°.

This leaf response seems to be an adaptation to low phosphate availability. Pi deficiency inhibits photosynthesis (Dietz & Foyer. 1986), so inducing this erect leaf phenotype would reduce light capture, thereby conserving Pi (Ruan et al., 2018).

3.1.2. Sweet Corn (Maize)



Figure 6. Visual representation of the effect of phosphate status on *Zea mays L* leaf inclination. A) 12 day old seedling grown under sufficient phosphate. B) 12 day old seedling grown under insufficient phosphate.

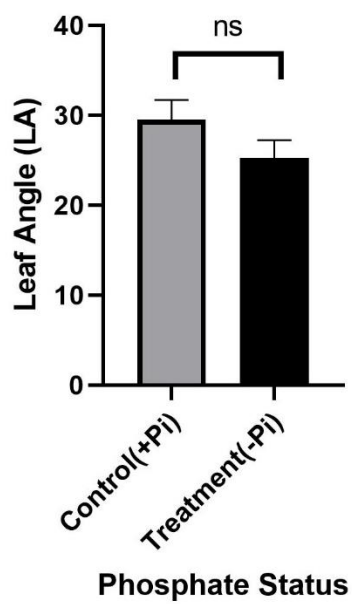


Figure 7. The effect of phosphate status on leaf inclination of *Zea mays L*. Values indicated are mean leaf angles + SEM ($n=16$). Asterisks indicate the degree of significant difference.

Starving sweet corn seedlings of phosphate resulted in a decrease in leaf inclination of flag leaves (Figures 6 and 7). However, this result was not statistically significant, and the average leaf angles of 29° and 25° were observed under sufficient phosphate and deficient phosphate conditions, respectively.

Sweet corn may show a stronger leaf inclination response to phosphate starvation. However, this may only become apparent later in its growth stage. One control of these growth experiments was to sample primary leaf inclination following development of the secondary leaf. A change in phenotype might occur in later developing tertiary or quaternary leaves, so sampling leaf inclination after a longer period of plant development would give a better understanding of the phosphate stress response.

3.1.3. Wheatgrass

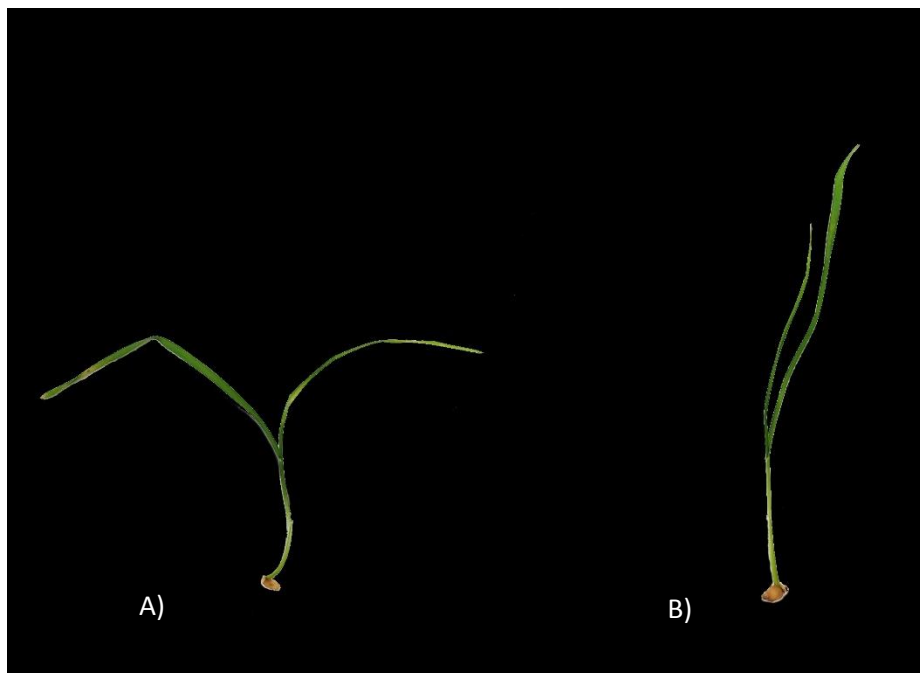


Figure 8. Visual representation of the effect of phosphate status on *Triticum aestivum* leaf inclination. A) 14 day old seedling grown under sufficient phosphate. B) 14 day old seedling grown under insufficient phosphate.

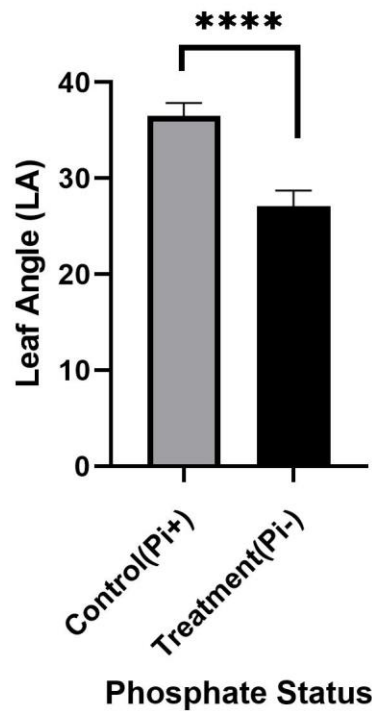


Figure 9. The effect of phosphate status on leaf inclination of *Triticum aestivum*. Values indicated are mean leaf angles + SEM (n=19). Asterisks indicate the degree of significant difference.

Also in agreement with the findings of a previous study (Ruan et al., 2018), phosphate starvation induced smaller primary leaf angles in wheatgrass than under sufficient phosphate conditions (Figures 8 and 9). An average leaf angle of 36° was observed under sufficient phosphate supply, while under insufficient phosphate supply, an average leaf angle of 27° was observed.

Wheatgrass demonstrated a greater significant difference in leaf inclination relative to Italian ryegrass (Figures 5 and 9), and it was, therefore, chosen as the model plant for all the other assays in the present study.

3.2 Cross section of *Triticum aestivum* lamina Joint

3.2.1. Transverse Lamina joint cross section under differing phosphate status

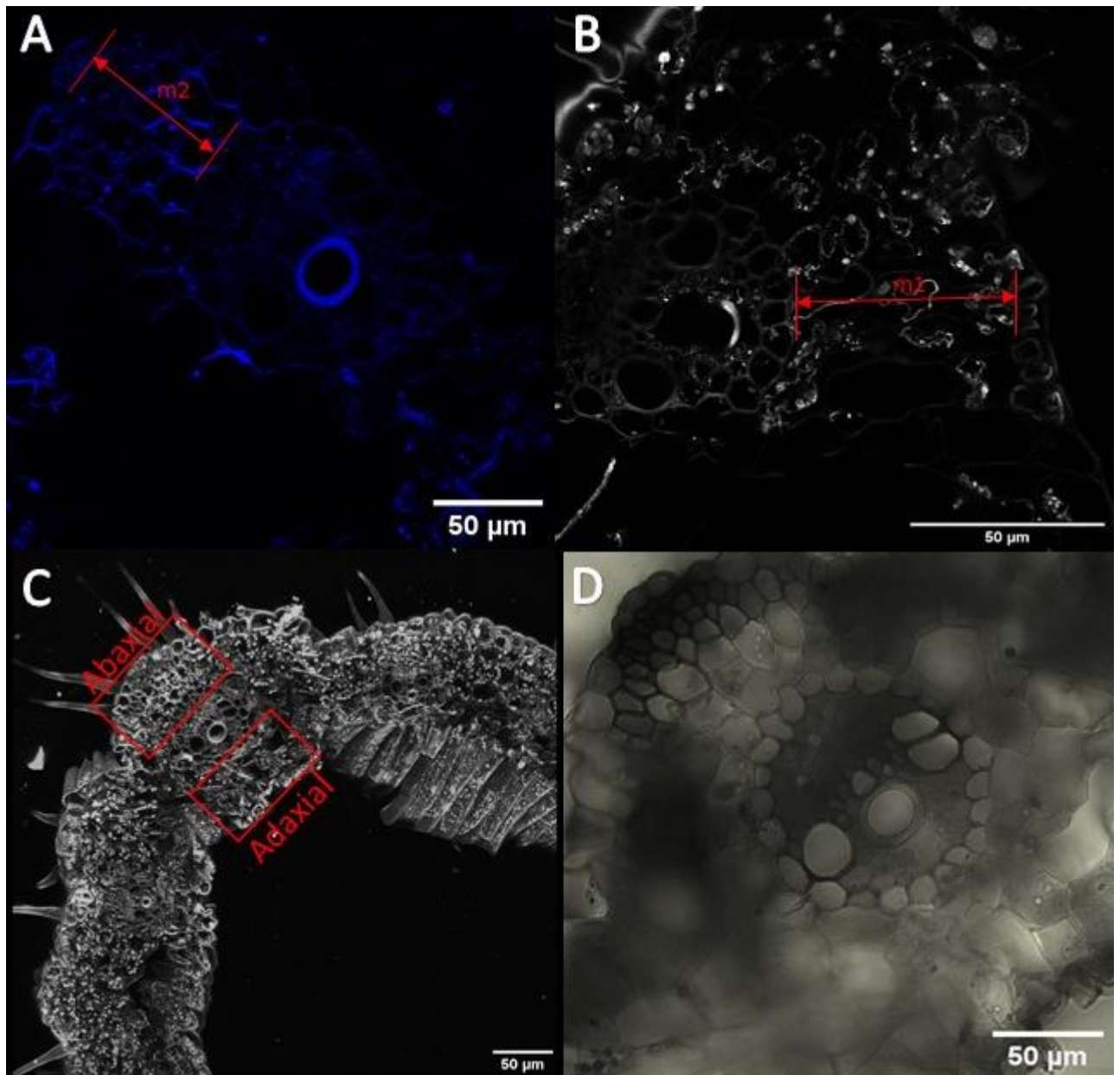


Figure 10. Transverse cross section of *Triticum aestivum* lamina joint developed under insufficient phosphate. a) Fluorescent image of the abaxial side of the lamina joint. m2 indicates the region between the abaxial epidermis and the central vascular bundle. b) Fluorescent image of the adaxial side of the lamina joint. m1 indicates the region between the adaxial epidermis and the central vascular bundle. c) Fluorescent 3D projection image of lamina joint. Red boxes indicate abaxial and adaxial sides of lamina joint, respectively. d) Bright field image of lamina joint. Scale bars=50µm

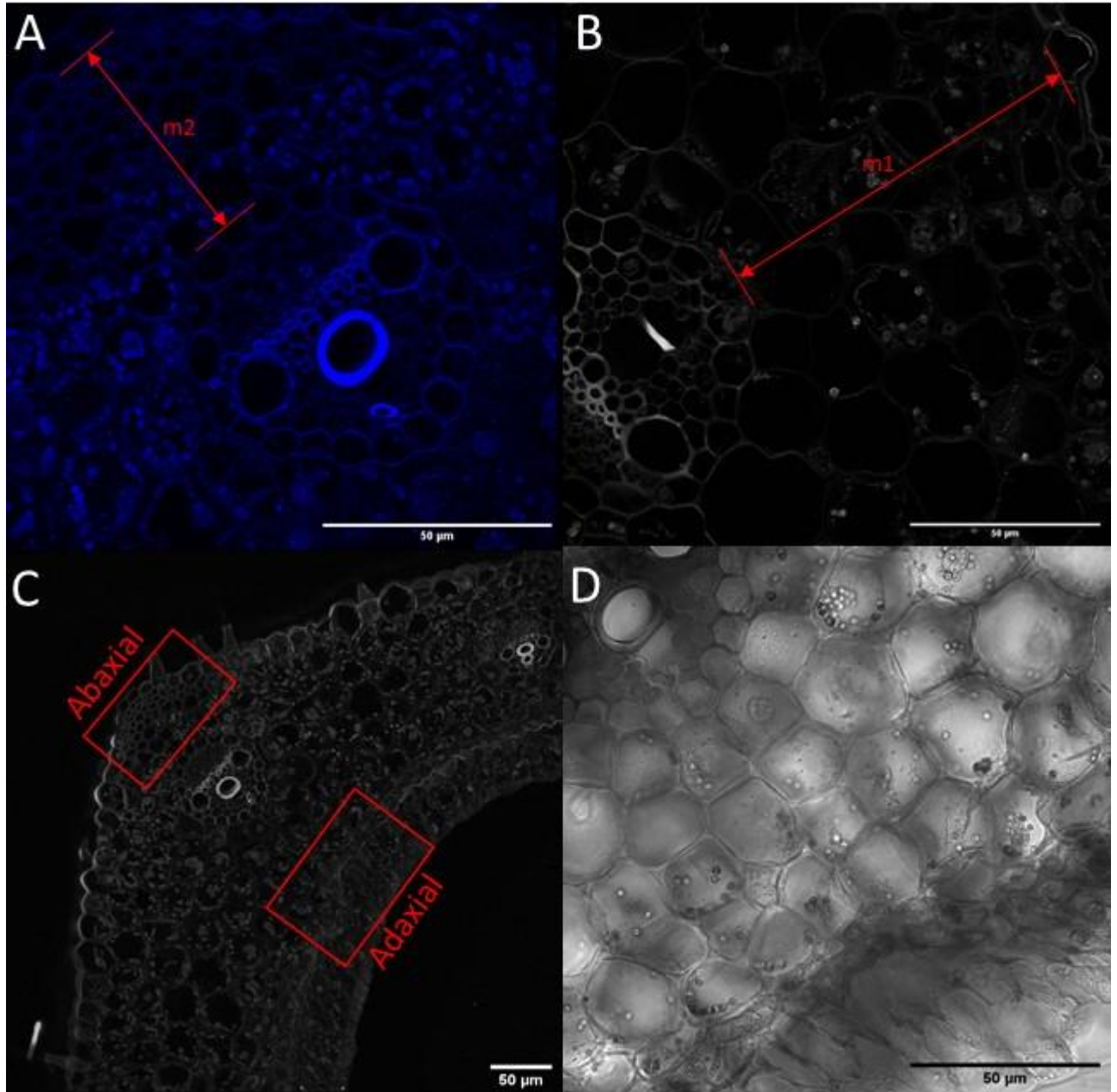


Figure 11. Transverse cross section of *Tricum aestivum* lamina joint developed under sufficient phosphate. a) Fluorescent image of the abaxial side of the lamina joint. m2 indicates the region between the abaxial epidermis and the central vascular bundle. b) Fluorescent image of the adaxial side of the lamina joint. m1 indicates the region between the adaxial epidermis and the central vascular bundle. c) Fluorescent 3D projection image of lamina joint. Red boxes indicate abaxial and adaxial sides of lamina joint, respectively. d) Bright field image of adaxial side of lamina joint. Scale bars=50µm

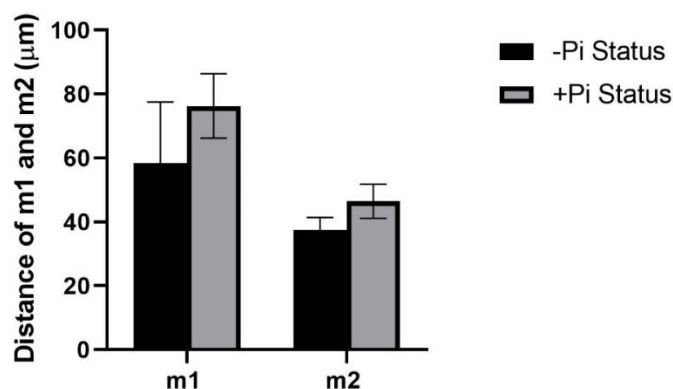


Figure 12. Measurement of the width of m1 and m2 regions under -Pi and +Pi status (shown in Figures 10a, 10 b and Figures 11a, and 11 b). Values indicated are the mean region width+ SEM (n=3). Asterisks indicate the degree of significant difference.

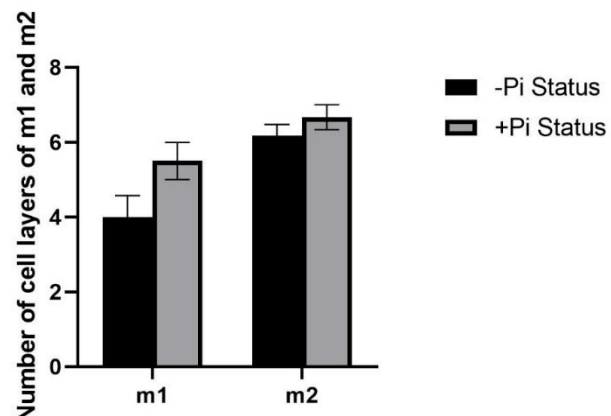


Figure 13. The number of cell layers in m1 region and sclerenchyma cell layers in m2 region under -Pi and +Pi status. (shown in Figures 10a, 10 b and Figures 11a, and 11 b). Values indicated are the mean number of cell layers+ SEM (n=3). Asterisks indicate the degree of significant difference.

Transverse cross sections of the *Triticum aestivum* lamina joint under insufficient (Figure 10) and sufficient phosphate (Figure 11) showed no significant difference in abaxial or adaxial region width (as shown in Figure 12). Additionally, there was no significant difference in the number of cell layers in m1 and m2 regions (Figure 13).

These findings contrast with observations of Ruan et al (2018). They demonstrated that phosphate stress inhibited cell proliferation and expansion in rice abaxial and adaxial lamina joint regions, reflected by reduced cell layers and width of m1 (Their m1 region was classified as the distance between the adaxial epidermis and sclerenchyma) and m2 (relative to +Pi conditions).

In this study, Indifference in m1 and m2 cell layer and width under differing phosphate status could be due to the variation present in such a small number of replicates completed (n=3). Ruan et al (2018) utilised 15 replicates for measuring the same regions.

In any case, the present findings suggest that leaf inclination in *Triticum aestivum* is regulated by another unknown cellular mechanism. It is possible that phosphate stress induces leaf erectness by reducing length of abaxial (sclerenchyma) and adaxial cells, relative to +Pi conditions (as was observed in Ruan et al., 2018). To investigate this, it would be necessary to produce longitudinal cross sections of the lamina joint; however, this was not possible within the timeframe of this study.

3.3 Interaction between phosphate status and PGRs on leaf inclination (*Triticum aestivum*)

3.3.1. Control experiment (ddH₂O)

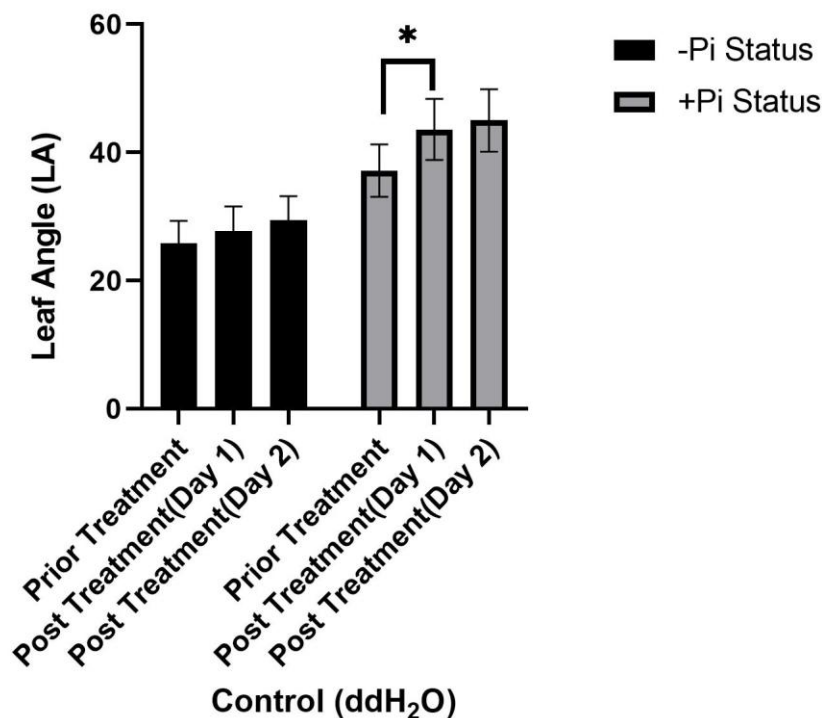


Figure 14. The effect of 48 h double distilled water incubation on leaf inclination of seedlings grown under differing phosphate status. Values indicated are mean leaf angles + SEM ($n=4$). Asterisks indicate the degree of significant difference.

In a previous study (Maeda. 1970), it was found that when excised rice lamina joints were placed in water, a gravitropic effect, causing an increase in leaf inclination was observed. In this study, excised wheatgrass segments also seemed to show a slight gravitropic response when placed in water. While increases in lamina inclination were observed under –Pi and +Pi status, only seedlings grown under +Pi showed a statistically significant increase during the first 24 h (Figure 14). This gravitropic response seems to be induced within a short timeframe, where another 24 h of incubation did not appear to further increase leaf inclination.

Therefore, to account for any lamina inclination increase due to the gravitropic response, excised leaf segments were incubated in double distilled water for 24 h prior to exposure to hormone treatments (the protocol used in phosphate status-nutrient interaction experiments was described in section 2.6).

3.3.2. Auxin experiments (IAA)

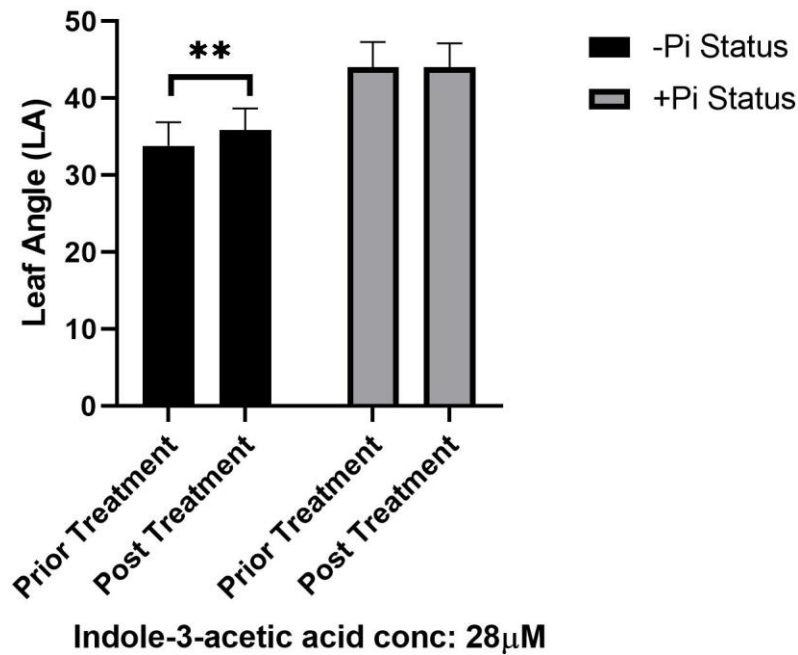


Figure 15. The effect of 48 h exogenous IAA incubation on leaf inclination of seedlings grown under differing phosphate status. Values indicated are mean leaf angles + SEM (n=4). Asterisks indicate the degree of significant difference.

Upon incubation of excised leaf segments from –Pi and +Pi seedlings in IAA, there was a slight increase in leaf inclination under insufficient phosphate, while no increase was observed under sufficient phosphate (Figure 15). Over the incubation period, the leaf angle of –Pi seedlings only increased by approximately 2°. Previous literature has demonstrated that auxin plays a role in the regulation of leaf inclination, (Chen et al., 2018; Liu et al., 2019; Zhao et al., 2013). Based on these current findings, exogenous application of IAA may help recover leaf inclination under phosphate stress, however the increase in leaf inclination is fairly minor.

Based on similar bioassays that looked at lamina inclination in rice, our results share similarities. Wada et al (1984) found that application of IAA to excised seedlings resulted in a minor increase in leaf inclination. However, a larger difference was only observed at an extremely high concentration of 50 mg/L. In contrast, Maeda (1965) observed that IAA concentrations as low as 5×10^{-6} M resulted in roughly a 50% increase in lamina inclination. These differences could potentially be attributed to variation in the cultivars used.

3.3.3. Absciscic acid experiments (ABA)

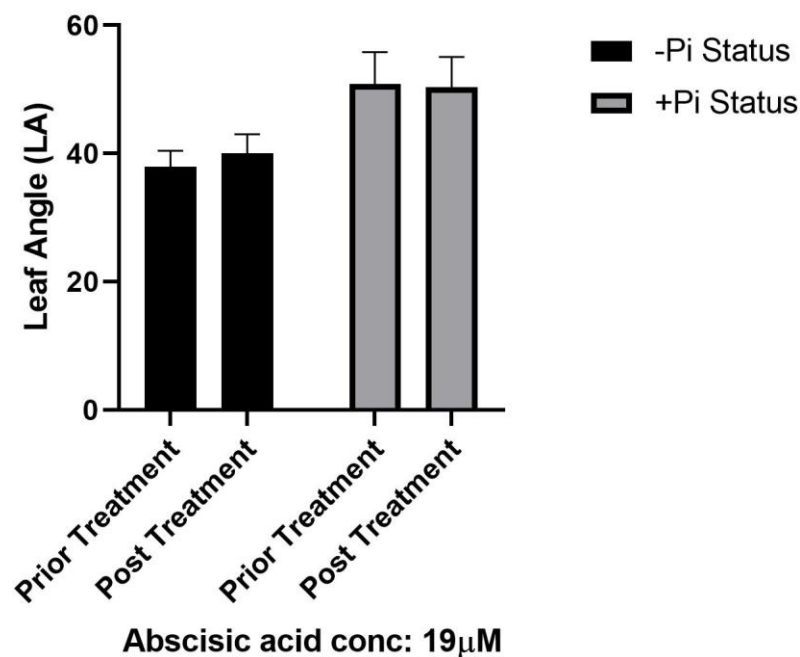


Figure 16. The effect of 48h exogenous ABA incubation on leaf inclination of seedlings grown under differing phosphate status. Values indicated are mean leaf angles + SEM (n=4). Asterisks indicate the degree of significant difference.

Incubation of wheatgrass seedlings in abscisic acid had no observable effect on lamina inclination, in either phosphate state (Figure 16). This is inconsistent with previous findings found in rice lamina inclination. Li et al (2019) found that leaf angle decreased following treatment with ABA, however this decrease required a concentration of 5 mM and higher. It is possible we did not see a decrease in lamina inclination, as our seedlings were grown under a 24 hour photoperiod, while in the majority of previous studies, rice lamina inclination studies were tested on etiolated seedlings (Cao & Chen. 1995; Fujii & Saka. 2001; Maeda. 1965; Wada et al., 1984). Etiolated lamina joints may be more sensitive to exogenous abscisic acid treatments.

3.3.4. Cytokinin experiments (BA)

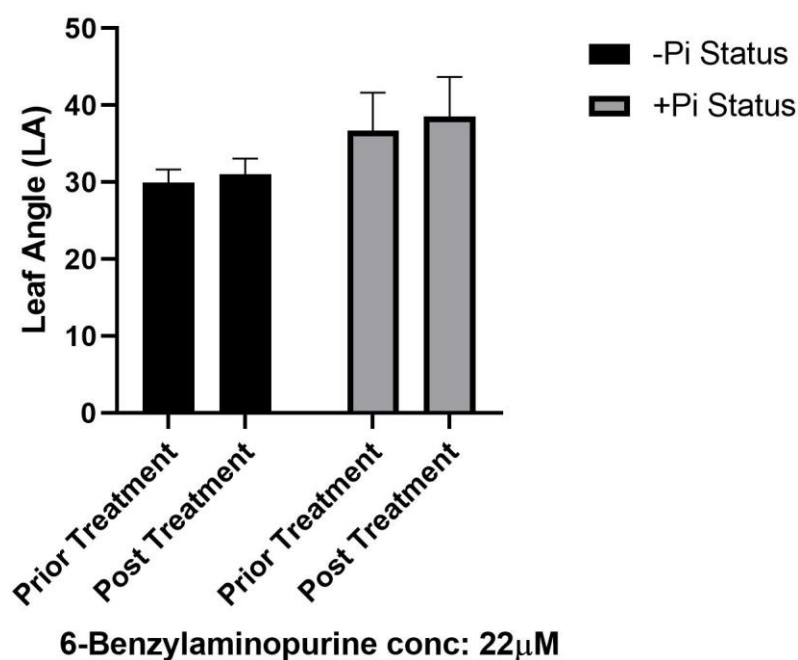


Figure 17. The effect of 48 h exogenous BA incubation on leaf inclination of seedlings grown under differing phosphate status. Values indicated are mean leaf angles + SEM (n=4). Asterisks indicate the degree of significant difference.

Incubation of seedlings in 6-benzylaminopurine had no significant effect on lamina inclination, regardless of phosphate status (Figure 17). This contrasts with the findings of Maeda (1965) , showing that an analogue of BA, kinetin, reduced lamina inclination of rice seedlings by 23% (relative to the control) at a low concentration of 2.5 μ M. Interestingly, a similar bioassay carried out by Wada et al (1984), observed that kinetin had weak inhibition of lamina inclination, at a relatively high concentration of 5 μ g/mL(a similar concentration as used in this study).

3.3.5. Brassinosteroid experiments (EBL application and inhibition)

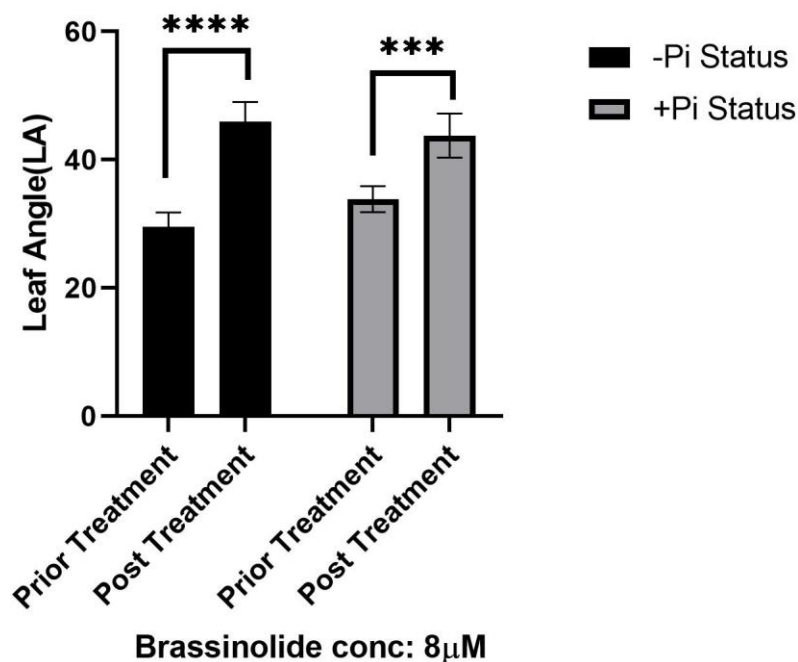


Figure 18. The effect of 48 h exogenous BL incubation on leaf inclination of seedlings grown under differing phosphate status. Values indicated are mean leaf angles + SEM (n=4). Asterisks indicate the degree of significant difference.

Consistent with previous findings, exogenous application of brassinolide significantly increased lamina inclination in seedlings grown under insufficient and sufficient phosphate (Figure 18). Brassinosteroids seem to be able to rescue phosphate deficient plants from their erect like phenotype; however, there was little difference in the response of seedlings based on phosphate status, which suggests that brassinosteroids may regulate lamina inclination through a different mechanism than phosphate supply.

Interestingly, in the previous work by Cao and Chen (1995), it was found that etiolated lamina had increased sensitivity to brassinolide than green lamina. They observed approximately a four-fold increase in lamina joint inclination in etiolated joints upon exposure to 1 μ M brassinolide.

Presently, only a 2-fold increase in leaf inclination was observed in *Triticum aestivum* green lamina grown under insufficient phosphate, and at a significantly higher concentration of brassinolide, relative to the protocol in Cao and Chen (1995).

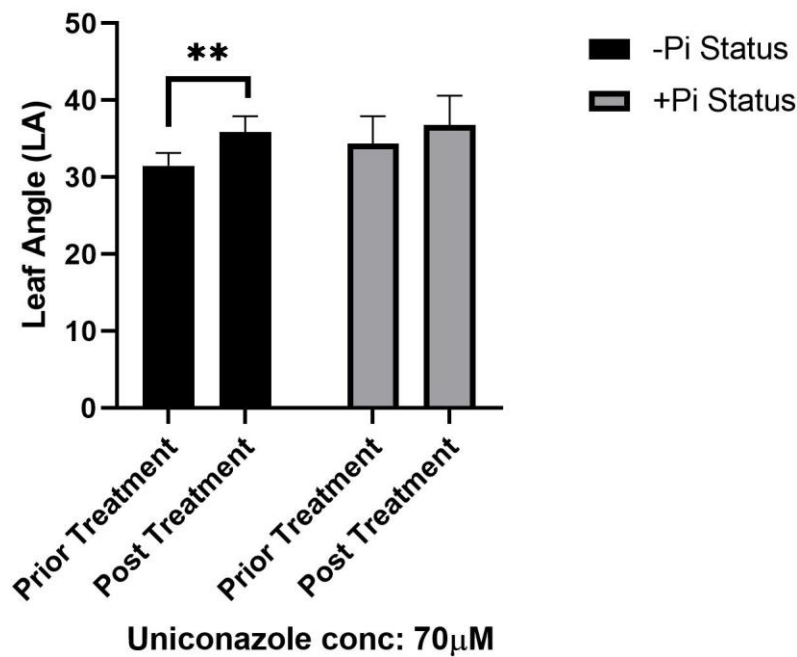


Figure 19. The effect of 48 h UCZ incubation on leaf inclination of seedlings grown under differing phosphate status. *Values indicated are mean leaf angles + SEM (n=4). Asterisks indicate the degree of significant difference.*

Treatment of excised lamina joints with endogenous brassinosteroid inhibitor , uniconazole ,resulted in an increase in leaf inclination under insufficient phosphate (Figure 19). This finding is interesting, as uniconazole was expected to negatively regulate leaf inclination, due to its inhibitory effect on endogenous brassinosteroid biosynthesis.

This result could potentially be attributed to the non-specificity of uniconazole's action, where it also interferes with biosynthesis of gibberellin (Rozhon et al., 2019). Additionally, uniconazole has shown strong inhibition of ABA catabolism in *Arabidopsis* (Saito et al., 2006).

Gibberellin may not participate in the negative regulation of lamina inclination, as Maeda (1965) observed that gibberellin increased leaf inclination in excised rice leaves (although the increase was minor and required a very high concentration of 50mg/L). Additionally, reduced expression of SPINDLY, a negative regulator of gibberellin signalling in rice, resulted in increased lamina joint inclination (Shimada et al., 2006).

3.3.6. Ethylene experiments (ET application and inhibition)

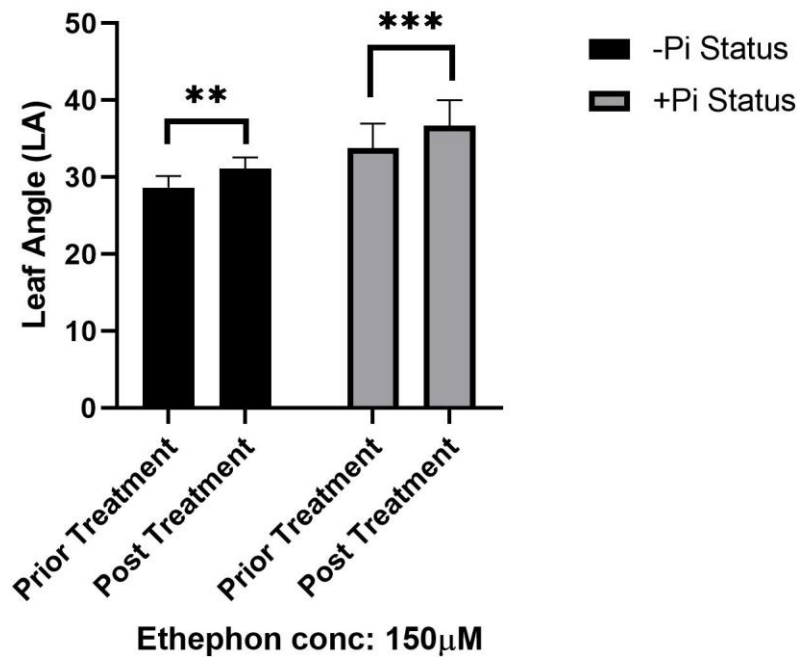


Figure 20. The effect of 48 h exogenous ET incubation on leaf inclination of seedlings grown under differing phosphate status. Values indicated are mean leaf angles + SEM (n=4). Asterisks indicate the degree of significant difference.

Application of ethylene precursor, ethephon, increased lamina inclination slightly under both insufficient and sufficient phosphate status (Figure 20). This suggests that ethylene may also act as a positive regulator of lamina inclination. Previously, Cao and Chen (1995) observed that an inhibitor of ethylene biosynthesis, CoCl_2 , inhibited BR-induced lamina inclination and ethylene production in rice. Additionally, it has been demonstrated that BR can induce ethylene production in tomato leaves (Schlagnhauser & Arteca. 1985).

Ethylene and brassinosteroid may interact to positively regulate lamina inclination, where they often display hormonal crosstalk (Jiroutova et al., 2018; Papadopoulou & Grumet. 2005; Shi et al., 2015), but further investigation is necessary.

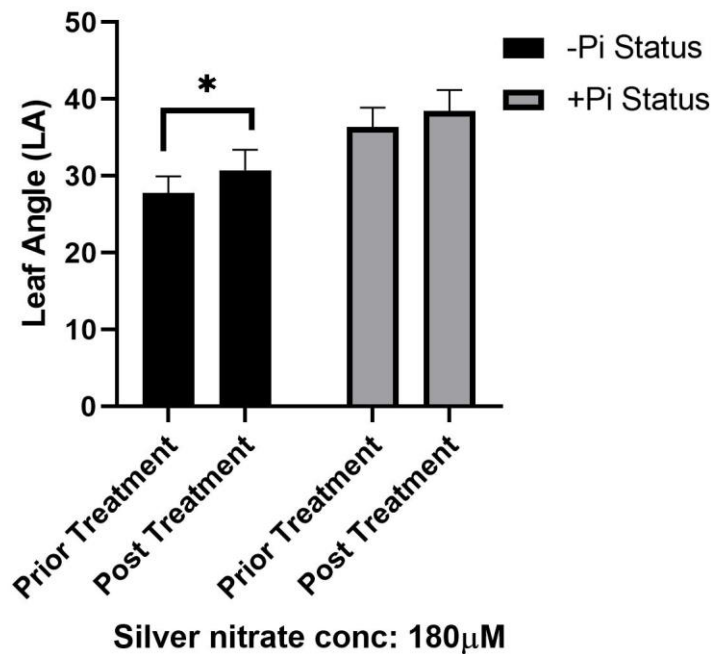


Figure 21. The effect of 48 h AgNO₃ incubation on leaf inclination of seedlings grown under differing phosphate status. Values indicated are mean leaf angles + SEM (n=4). Asterisks indicate the degree of significant difference.

Upon treatment of lamina joints with AgNO₃, there was an increase in leaf inclination under insufficient phosphate (Figure 21). This finding is interesting, considering that AgNO₃'s site of action is at the ethylene receptors, where silver modulates the binding site, and prevents the transduction of the ethylene signal (Schaller & Binder. 2017). These findings seem to contradict earlier observations, as applying ethylene increased lamina inclination (Figure 20).

However, silver nitrate is a non-specific inhibitor, as it has been found to have off-target effects on auxin activity, through stimulating auxin efflux (Strader et al., 2009). It is plausible that the increase in leaf inclination could be due to off-target activity of silver nitrate.

3.4. Effect of phosphate status on lamina joint ROS production (*Triticum aestivum*)

3.4.1 Superoxide assay (Qualitative)



Figure 22. Superoxide production in *Triticum aestivum* lamina joint under differing phosphate status. A1) +Pi seedling following 2 hours under exposure to NBT. A2) +Pi seedling visualised under a stereo microscope. B1) -Pi seedling following 2 hours under NBT exposure. B2) -Pi seedling visualised under a stereo microscope. (n=3)

Following infiltration of NBT over 2 hours, there clearly appears to be a difference in the production of superoxide between seedlings grown under different phosphate supply (Figure 22). Production of superoxide was evident at the segment tips, which was due to wounding, however, more importantly, there were differences in superoxide production in the lamina joint (observed in A2 and B2). There was significantly more superoxide produced under

insufficient phosphate, where superoxide seemed to be localized at the marginal, adaxial side of lamina joint, and at the auricle.

3.4.2. Hydrogen peroxide assay (Qualitative)

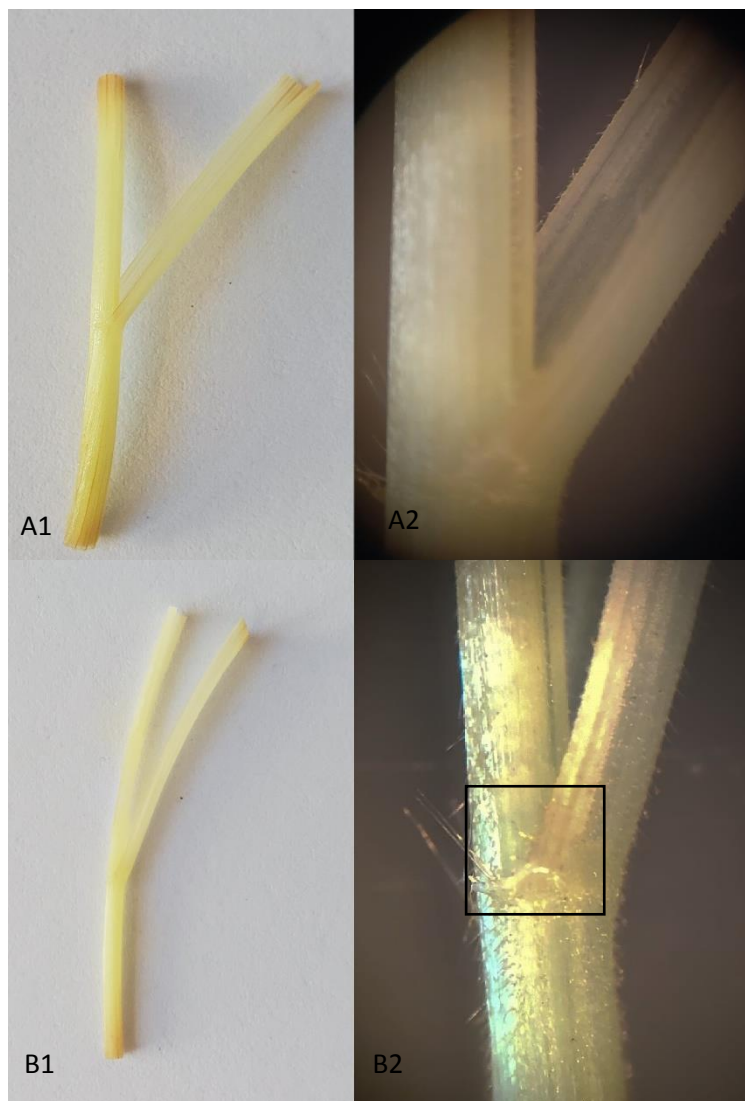


Figure 23. Hydrogen peroxide production in *Triticum aestivum* lamina joint under differing phosphate status. A1) +Pi seedling following 2 hours under exposure to DAB. A2) +Pi seedling visualised under a stereo microscope. B1) -Pi seedling following 2 hours under DAB exposure. B2) -Pi seedling visualised under a stereo microscope. (n=3)

Upon exposure to DAB, there seems to be a difference in hydrogen peroxide production under varying phosphate status (Figure 23), with more hydrogen peroxide observed in the lamina region under insufficient phosphate. Consistent with superoxide production, hydrogen peroxide also appeared to be localized at the marginal adaxial side of lamina joint, with stain also observed at the auricle. Observing the stain was somewhat difficult, due to the light red pigmentation of DAB stain against the yellow colour of the bleached seedlings.

Based on the similar localization of both stains, it is possible that hydrogen peroxide and/or superoxide trigger a phosphate deficiency stress induced signalling response at the marginal adaxial side of the lamina joint.

3.4.3. Peroxidase assay (Quantitative)

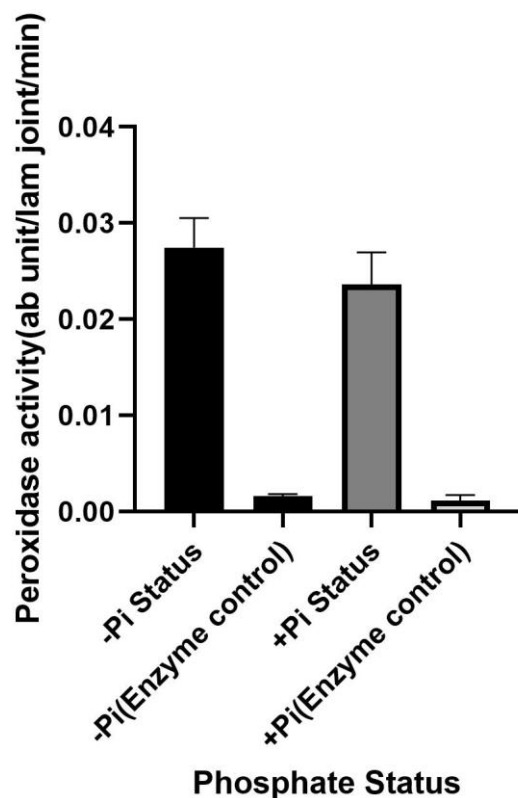


Figure 24. Peroxidase activity of lamina joint under differing phosphate status. *Values indicated are the mean absorbance/lamina joint/minute + SEM (n=4). Asterisks indicate the degree of significant difference*

Seedlings grown under differing phosphate status had no significant difference in guaiacol peroxidase activity, as shown in Figure 24. This finding is interesting, considering that guaiacol peroxidase is well accepted as a stress responsive enzyme in the defence against biotic and abiotic stress (Sharma et al., 2012). Peroxidase activity has been repeatedly shown to increase

under abiotic stress in order to scavenge ROS, which can cause damage through lipid peroxidation of membranes, along with oxidative damage to proteins, DNA and RNA (Choudhury et al., 2017; Mittler. 2002).

There is also a fine balancing act between both ROS formation, and the upregulation of peroxidases and a whole battery of other antioxidant and non-enzymatic antioxidants (Waskiewicz et al., 2014). Indifference in our observed peroxidase activity under differing phosphate status, could be due to guaiacol peroxidase having no major role in scavenging ROS, under phosphate stress. Another antioxidant not measured in this study, may respond more strongly to this form of abiotic stress.

Alternatively, this indifference could be due to the requirement for slightly increased ROS formation under phosphate stress, in order to negatively regulate leaf inclination. If scavenging activity of peroxidase was too great, this may limit the concentration of ROS, and thus inhibit the signal transduction ability of ROS.

3.4.4. Lipid peroxidation assay (Quantitative)

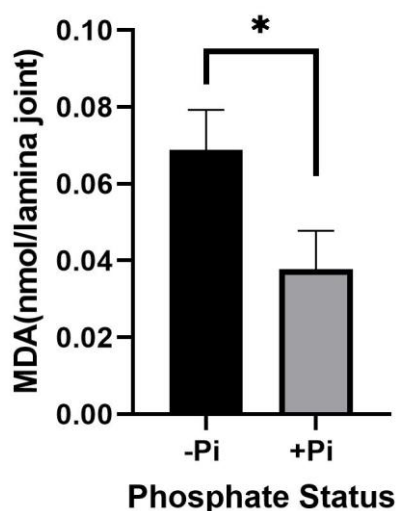


Figure 25. MDA content of lamina joint under differing phosphate status. *Values indicated are the mean amount of MDA in nmol/lamina joint + SEM (n=4). Asterisks indicate the degree of significant difference.*

Lipid peroxidation was quantified by measuring the content of lipid peroxidation end product, malondialdehyde.

Increased lipid peroxidation was observed in the lamina joint region of seedlings grown under insufficient phosphate (Figure 25), relative to sufficient phosphate. This quantitative finding supports our qualitative observations which were discussed earlier (Figures 22 and 23).

Relating this to the peroxidase activity discussed earlier (Figure 24), this suggests that there could be an imbalance between ROS formation and ROS scavenging, with a higher ratio of ROS formation/ROS scavenging under insufficient phosphate.

With a higher level of ROS formation under phosphate stress, this would have to be maintained at a non-toxic level, in order for ROS to be utilized as signalling molecules (Deng et al., 2016).

3.4.5. Lipid peroxidation assay on seedlings grown with exogenous brassinolide

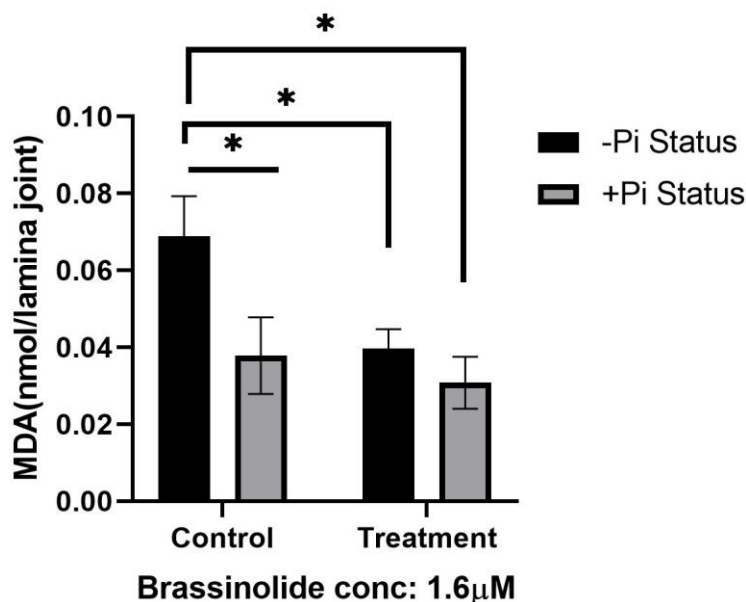


Figure 26. MDA content of lamina joint under differing phosphate status and exogenous BR. Values indicated are the mean amount of MDA in nmol/lamina joint + SEM (n=4). Asterisks indicate the degree of significant difference.

Upon exogenous brassinolide treatment, there was significant reduction in lipid peroxidation under insufficient phosphate (Figure 26), reaching similar MDA content levels to seedlings grown under sufficient phosphate. Further reduction in MDA content from brassinolide treatment was observed under sufficient phosphate, however this was non-significant.

Brassinosteroids are well known to be important in ameliorating the effect of various abiotic and biotic stresses. This is achieved through their scavenging ability of ROS by the modulation of various antioxidant responses (Bajguz & Hayat. 2009; Vardhini & Anjum. 2015). For example, application of homo-brassinolide improved cadmium tolerance in *Brassica juncea* by increasing activity of antioxidative enzymes, catalase, peroxidase, and superoxide dismutase (Hayat et al., 2007).

Based on these present findings, this suggests that brassinosteroid can ameliorate the effect of phosphate stress, through negative regulation of ROS. Additionally, BR also positively regulates leaf inclination under phosphate stress (Figure 18), so it seems to have a multi-level regulatory role in the leaf inclination response. Further investigation of phosphate-brassinosteroid interactions is required to elucidate this regulatory mechanism.

To confirm whether brassinosteroid modulates antioxidant activity under phosphate stress, it would be necessary to assay the activity of enzymes such as peroxidase and catalase (this was not achievable within the timeframe of this study).

3.5. Effect of exogenous NO on leaf inclination (*Triticum aestivum*)

3.5.1 Effect of SNP treatment on leaf inclination under differing phosphate status

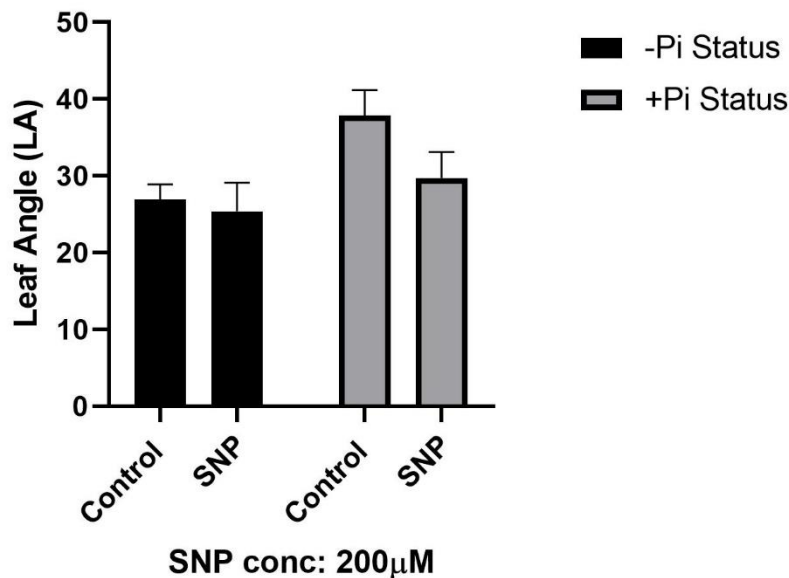


Figure 27. The effect of SNP (200µM) treatment on leaf inclination under differing phosphate status. Values indicated are the mean leaf angles + SEM (n=5). Asterisks indicate the degree of significant difference.

To investigate the potential role of NO in regulation of phosphate induced leaf inclination, NO donor, SNP, was applied exogenously.

A decrease in leaf inclination was observed under sufficient phosphate; however, this difference was not significant (Figure 27). The effect of SNP was also tested by a seed pre-

treatment protocol, which also returned non-significant results (Figure S1). This suggests that NO does not play a role in the negative regulation of leaf inclination.

Alternatively, our non-significant results could possibly be explained by our assays simply lacking sensitivity. For example, one difficulty in utilising SNP as an NO donor, is its light sensitivity, where it degrades rapidly upon exposure to light, along with releasing cyanide ions (Arnold et al., 1984; Bisset et al., 1981).

Under the light intensity of our growth room, rapid degradation of SNP is likely, leading to a quick release of NO. Since development of the primary lamina joint occurs approximately 12 days from germination (under the present growth conditions), there may be little NO present to modulate lamina joint development at this stage of growth.

3.5.2. Effect of SNP and cPTIO treatment on leaf inclination under differing phosphate status

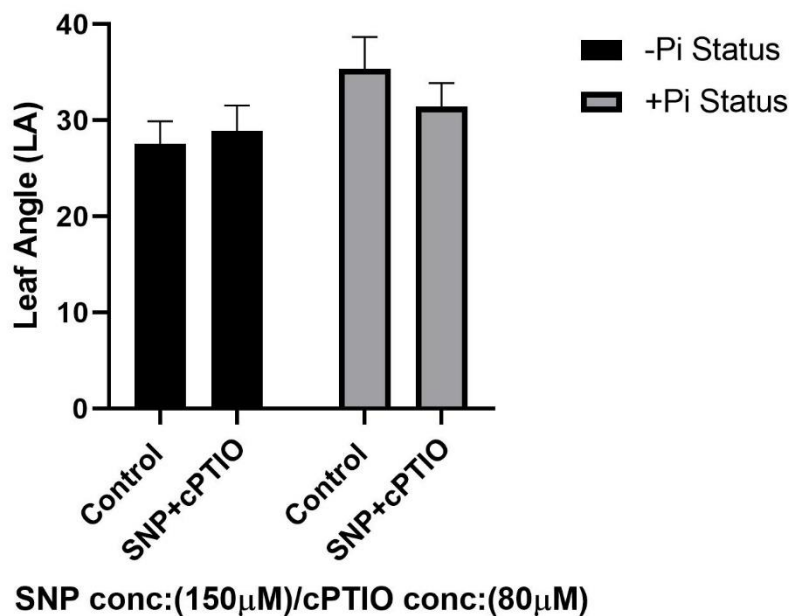


Figure 28. The effect of SNP(150 μ M) and cPTIO (80 μ M) treatment on leaf inclination under differing phosphate status. *Values indicated are the mean leaf angles + SEM (n=5). Asterisks indicate the degree of significant difference.*

To determine that any change in leaf inclination was due to the NO donor activity of SNP, and not due to cyanide ion release, SNP was applied along with NO scavenger, cPTIO. There was no significant difference in leaf inclination following treatment under either insufficient or sufficient phosphate (Figure 28). Similar results were obtained using a seed pre-treatment protocol using 100 μ M SNP and 100 μ M cPTIO (Figure S2).

While a non significant decrease in leaf inclination is observed under sufficient phosphate, the degree of this decrease is smaller relative to under SNP treatment (Figure 27). In any case, these results are consistent with observations from the SNP treatment.

3.5.3. Effect of cPTIO seed pre-treatment on leaf inclination under differing phosphate status

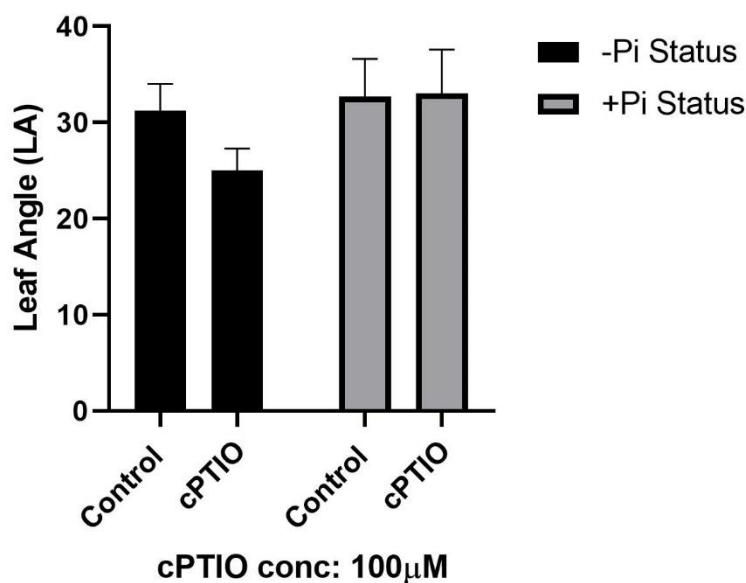


Figure 29. The effect of cPTIO (100µM) seed pre-treatment on leaf inclination under differing phosphate status. Values indicated are the mean leaf angles + SEM (n=5). Asterisks indicate the degree of significant difference.

To aid as another control for testing SNP activity, the effect of scavenging NO on leaf inclination was examined by pre-treating seeds with 100µM cPTIO. cPTIO pre-treatment had no significant effect on leaf inclination, while there was a non significant decrease in leaf inclination under insufficient phosphate (Figure 29).

Applying cPTIO through a seed pre-treatment protocol also lacks sensitivity, as this is unlikely to impact lamina joint development, which occurs approximately 12 days from sowing. Many protocols that involve SNP and/or cPTIO pre-treatment are investigating growth characteristics shortly after germination (Phang et al., 2011; Rather et al., 2020; Zhou et al., 2012).

4. Final Conclusions and Future Directions

4.1 Key Findings

The aim of this research was to determine whether cereal crops, maize (*Zea mays* L) and wheatgrass (*Triticum aestivum*), and a forage crop, Italian ryegrass (*Lolium multiflorum*) would show a decrease in leaf inclination under insufficient phosphate supply. The findings from this study demonstrated that both Italian ryegrass and wheatgrass responded to phosphate stress by reducing leaf inclination through the negative regulation of the lamina joint, with wheatgrass showing the greatest reduction in leaf inclination.

Transverse cross sections of the *Triticum aestivum* lamina joint showed no difference in abaxial and adaxial region width or cell layers under differing phosphate status. The cellular mechanism by which phosphate stress regulates leaf inclination clearly remains to be determined.

The interaction between phosphate supply and hormones in regulation of *Triticum aestivum* leaf inclination was also investigated. Brassinosteroid had the strongest effect on leaf inclination, increasing leaf angle under both insufficient and sufficient phosphate status, while ethylene also increased leaf inclination marginally. Application of auxin slightly increased leaf inclination under insufficient phosphate status, suggesting auxin may be able to partially counteract the negative regulatory action of phosphate deficiency. It remains largely unclear how phosphate status interacts with hormones to regulate leaf inclination.

This study also found considerable evidence (qualitative and quantitative) to suggest increased production of ROS under phosphate stress. Under phosphate stress, *Triticum aestivum* seedlings showed increased production of superoxide and hydrogen peroxide in the marginal adaxial side of the lamina joint. This was confirmed by increased lipid peroxidation end product, malondialdehyde, in lamina joint segments from seedlings grown under insufficient phosphate. These findings strongly suggest that ROS is an important signalling molecule in the negative regulation of leaf inclination under phosphate stress. Additionally, exogenous application of brassinosteroid, was able to reduce lipid peroxidation in seedlings grown under phosphate stress. This suggests that brassinosteroid may be involved in crosstalk with ROS, such as hydrogen peroxide, where it downregulates ROS production to positively regulate leaf inclination.

Based on the SNP treatment, NO did not appear to have a signalling role in regulation of leaf inclination under phosphate stress. However, the sensitivity of the current protocol is questionable. Detecting intracellular NO will be critical to determine whether NO has any relevant signalling role in this physiological response.

These findings demonstrate that other important crops such as Italian ryegrass and wheatgrass (besides rice), have developed a mechanism to respond to phosphate stress, by

reducing leaf inclination. ROS seem to be important signalling molecules in inducing this physiological response.

Understanding how phosphate levels affect leaf inclination potentially has industrial value, where this knowledge could assist in efforts for selectively breeding plants with greater phosphate use efficiency, improve crop yields by maximising light interception down through the canopy, and potentially limit the application of phosphate based fertilisers.

4.2 Limitations of Study

The growth environment that the cereal and forage plants were exposed to was suboptimal. As seedlings were grown under constant light, this meant that light damage became a potential issue, where noticeable negative effects such as leaf chlorosis, and necrosis are possible (Velez-Ramirez et al., 2011). Several adjustments to the light intensity were made, including reducing output intensity, and increasing distance between light source and seedlings. Increasing the distance, meant seedlings grew more spindly, where seedling leaves quickly reached the lid of growth jars, and thus restricted growing space for developing seedlings. It is unclear how this may influence leaf inclination of primary leaves.

The adapted hormone incubation assay used in this study, seems to lack sensitivity, considering that the majority of previous studies investigated hormone responses in etiolated seedlings, rather than in green lamina (Fujii & Saka. 2001; Maeda. 1965; Wada et al., 1984). An alternative approach for determining hormone-nutrient interactions in leaf inclination is needed, for example, supplementation of growth media with hormone of interest during seedling development.

Both peroxidase and lipid peroxidation assays utilised, lack sensitivity. Guaiacol also reacts with phenolases such as tyrosinase and laccase, which negatively impacts the accuracy of the assay (Mirazizi et al., 2016). When measuring lipid peroxidation by the TBARS assay at a wavelength of 532nm, carbohydrates and anthocyanins interfere with measurements around this same wavelength (Taulavuori et al., 2001).

Results from the leaf inclination response to exogenous NO were inconclusive. This is potentially due to the rapid degradation of SNP upon exposure to light (which was present in our system). A more sensitive method used in Meng et al (2012), utilised NO donor, S-nitrosoglutathione (GSNO), which was supplied in the growth medium during plant development. Following a similar protocol would be beneficial for future evaluations of exogenous NO donor effects.

Due to the unforeseen circumstances of the COVID-19 pandemic, this led to many delays, and difficulties in shipping reagents (imported from overseas) required for experimental work. In particular, delays in the shipping of DAF-DM dye meant it was not possible to perform experiments in attempting to localize, and quantify the production of nitric oxide within the

timeframe of the masters project. As a result, there is insufficient evidence to suggest the role of NO as a signalling molecule in phosphate stress induced leaf inclination.

4.3 Future Research Suggestions

Both Italian ryegrass and wheatgrass have shown reduced primary leaf inclination under phosphate stress. However, it remains unclear how phosphate stress affects leaf inclination of later developing leaves (secondary and tertiary). A longer term growth assay could be conducted to investigate this physiological response.

Investigating effects of phosphate stress on leaf inclination under field conditions (*in situ*) would be necessary to determine how this response is affected by uncontrolled biotic and abiotic factors. Furthermore, this will help discern whether manipulating phosphate levels actually has industrial value, and can translate into increased crop yield.

Quantifying and localizing intracellular hydrogen peroxide and superoxide formation in lamina joints using sensitive fluorescent probes (such as CM-H₂DCFDA to detect H₂O₂, as used in Wang et al. (2014)) would be important assays to conduct in order to provide further evidence for the signalling role of these ROS under phosphate stress.

Further investigation is required to determine whether NO plays a signalling role in phosphate induced leaf inclination. This could be achieved by using a fluorescent dye, such as DAF-DM. It would be worthy of investigating the potential role of other common signalling molecules, such as calcium, in phosphate induced leaf inclination. Previous work by Yang and Komatsu (2000) found that a calcium dependent kinase was involved in leaf inclination caused by brassinolide.

Finally, it would be interesting to conduct genetic and protein interaction assays to discern whether an ortholog of the SPX protein (Ruan et al., 2018) is present in wheatgrass, and whether it regulates leaf inclination in a similar manner.

5. References

1. Arnold, W. P., Longnecker, D. E., & Epstein, R. M. (1984). Photodegradation of sodium nitroprusside: biologic activity and cyanide release. *Anesthesiology*, 61(3), 254-260. doi:10.1097/0000542-198409000-00004
2. Bajaj, S., Puthigae, S., Bryant, C., Whittaker, D., Elborough, K., & Hanley, Z. (2010). Ryegrass genes-a potential source for improving rice and grass species. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, 18(1), 69-71.
3. Bajguz, A., & Hayat, S. (2009). Effects of brassinosteroids on the plant responses to environmental stresses. *Plant Physiology and Biochemistry*, 47(1), 1-8. doi:10.1016/j.plaphy.2008.10.002
4. Baque, M. A., Lee, E. J., & Paek, K. Y. (2010). Medium salt strength induced changes in growth, physiology and secondary metabolite content in adventitious roots of *Morinda citrifolia*: The role of antioxidant enzymes and phenylalanine ammonia lyase. *Plant Cell Rep*, 29(7), 685-694. doi:10.1007/s00299-010-0854-4
5. Becraft, P. W., Bongard-Pierce, D. K., Sylvester, A. W., Poethig, R. S., & Freeling, M. (1990). The *liguleless-1* gene acts tissue specifically in maize leaf development. *Dev Biol*, 141(1), 220-232. doi:10.1016/0012-1606(90)90117-2
6. Bisset, W. I. K., Butler, A. R., Glidewell, C., & Reglinski, J. (1981). Sodium nitroprusside and cyanide release: Reasons for re-appraisal. *British Journal of Anaesthesia*, 53(10), 1015-1018. doi:10.1093/bja/53.10.1015
7. Bright, J., Desikan, R., Hancock, J. T., Weir, I. S., & Neill, S. J. (2006). ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H₂O₂ synthesis. *Plant Journal*, 45(1), 113-122. doi:10.1111/j.1365-313X.2005.02615.x
8. Cakmak, I. (2002). Plant nutrition research: Priorities to meet human needs for food in sustainable ways. *Plant and Soil*, 247(1), 3-24. doi:10.1023/a:1021194511492
9. Cao, H., & Chen, S. (1995). Brassinosteroid-induced rice lamina joint inclination and its relation to indole-3-acetic acid and ethylene. *Plant Growth Regulation*, 16(2), 189-196. doi:10.1007/BF00029540
10. Chen, S.-H., Zhou, L.-J., Xu, P., & Xue, H.-W. (2018). SPOC domain-containing protein Leaf inclination3 interacts with LIP1 to regulate rice leaf inclination through auxin signaling. *PLOS Genetics*, 14(11), e1007829. doi:10.1371/journal.pgen.1007829
11. Choudhury, F. K., Rivero, R. M., Blumwald, E., & Mittler, R. (2017). Reactive oxygen species, abiotic stress and stress combination. *Plant J*, 90(5), 856-867. doi:10.1111/tpj.13299
12. Cvetkovska, M., Alber, N. A., & Vanlerberghe, G. C. (2013). The signaling role of a mitochondrial superoxide burst during stress. *Plant Signaling and Behavior*, 8(1), 161-166.
13. Deng, X. G., Zhu, T., Zou, L. J., Han, X. Y., Zhou, X., Xi, D. H., . . . Lin, H. H. (2016). Orchestration of hydrogen peroxide and nitric oxide in brassinosteroid-mediated systemic virus resistance in *Nicotiana benthamiana*. *Plant Journal*, 85(4), 478-493. doi:10.1111/tpj.13120
14. Desikan, R., Neill, S. J., & Hancock, J. T. (2000). Hydrogen peroxide-induced gene expression in *Arabidopsis thaliana*. *Free Radical Biology and Medicine*, 28(5), 773-778. doi:10.1016/s0891-5849(00)00157-x
15. Dietz, K. J., & Foyer, C. (1986). The relationship between phosphate status and photosynthesis in leaves - Reversibility of the effects of phosphate deficiency on photosynthesis. *Planta*, 167(3), 376-381. doi:10.1007/BF00391342
16. Duvick, D. N. (2005). The contribution of breeding to yield advances in maize (*Zea mays* L.). In D. L. Sparks (Ed.), *Advances in Agronomy, Volume 86* (Vol. 86, pp. 83-145). San Diego: Elsevier Academic Press Inc.

17. . Food Science and Technology: International Series. (2008). In I. S. Arvanitoyannis (Ed.), *Waste Management for the Food Industries* (pp. ii). Amsterdam: Academic Press.
18. Fujii, S., & Saka, H. (2001). The promotive effect of brassinolide on lamina joint-cell elongation, germination and seedling growth under low-temperature stress in rice (*Oryza sativa* L.). *Plant Production Science*, 4(3), 210-214. doi:10.1626/pp.4.210
19. Hayat, S., Ali, B., Aiman Hasan, S., & Ahmad, A. (2007). Brassinosteroid enhanced the level of antioxidants under cadmium stress in *Brassica juncea*. *Environmental and Experimental Botany*, 60(1), 33-41. doi:10.1016/j.envexpbot.2006.06.002
20. Hodges, D. M., DeLong, J. M., Forney, C. F., & Prange, R. K. (1999). Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta*, 207(4), 604-611. doi:10.1007/s004250050524
21. Hossain, M. A., Bhattacharjee, S., Armin, S. M., Qian, P., Xin, W., Li, H. Y., . . . Tran, L. S. (2015). Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging. *Front Plant Sci*, 6, 420. doi:10.3389/fpls.2015.00420
22. Jang, S., & Li, H. Y. (2017). *Oryza sativa* BRASSINOSTEROID UPREGULATED1 LIKE1 induces the expression of a gene encoding a small leucine-rich-repeat protein to positively regulate lamina inclination and grain size in rice. *Frontiers in Plant Science*, 8. doi:10.3389/fpls.2017.01253
23. Jiroutova, P., Oklestkova, J., & Strnad, M. (2018). Crosstalk between Brassinosteroids and Ethylene during Plant Growth and under Abiotic Stress Conditions. *International journal of molecular sciences*, 19(10), 3283. doi:10.3390/ijms19103283
24. Johnston, R., Wang, M., Sun, Q., Sylvester, A. W., Hake, S., & Scanlon, M. J. (2014). Transcriptomic Analyses Indicate That Maize Ligule Development Recapitulates Gene Expression Patterns That Occur during Lateral Organ Initiation. 26(12), 4718-4732. doi:10.1105/tpc.114.132688 %J The Plant Cell
25. Jones, P. G., & Thornton, P. K. (2003). The potential impacts of climate change on maize production in Africa and LatinAmerica in 2055. *Global Environmental Change-Human and Policy Dimensions*, 13(1), 51-59. doi:10.1016/s0959-3780(02)00090-0
26. Juszczuk, I., Malusa, E., & Rychter, A. M. (2001). Oxidative stress during phosphate deficiency in roots of bean plants (*Phaseolus vulgaris* L.). *Journal of Plant Physiology*, 158(10), 1299-1305. doi:10.1078/0176-1617-00541
27. Kong, F., Zhang, T., Liu, J., Heng, S., Shi, Q., Zhang, H., . . . Li, G. (2017). Regulation of Leaf Angle by Auricle Development in Maize. *Molecular Plant*, 10(3), 516-519. doi:10.1016/j.molp.2017.02.001
28. Kumar, D., Yusuf, M. A., Singh, P., Sardar, M., & Sarin, N. B. (2014). Histochemical Detection of Superoxide and H₂O₂ Accumulation in *Brassica juncea* Seedlings. *Bio-protocol*, 4(8), e1108. doi:10.21769/BioProtoc.1108
29. Lee, D. J., Choi, H. J., Moon, M. E., Chi, Y. T., Ji, K. Y., & Choi, D. (2017). Superoxide serves as a putative signal molecule for plant cell division: overexpression of CaRLK1 promotes the plant cell cycle via accumulation of O₂ – and decrease in H₂O₂. *Physiologia Plantarum*, 159(2), 228-243. doi:10.1111/ppl.12487
30. Lee, J., Park, J. J., Kim, S. L., Yim, J., & An, G. (2007). Mutations in the rice liguleless gene result in a complete loss of the auricle, ligule, and laminar joint. *Plant Molecular Biology*, 65(4), 487-499. doi:10.1007/s11103-007-9196-1
31. Liu, K., Cao, J., Yu, K., Liu, X., Gao, Y., Chen, Q., . . . Yao, Y. (2019). Wheat TaSPL8 Modulates Leaf Angle Through Auxin and Brassinosteroid Signaling. *Plant Physiology*, 181(1), 179-194. doi:10.1104/pp.19.00248
32. Lobell, D. B., Hammer, G. L., McLean, G., Messina, C., Roberts, M. J., & Schlenker, W. (2013). The critical role of extreme heat for maize production in the United States. *Nature Climate Change*, 3(5), 497-501. doi:10.1038/nclimate1832

33. Lopez-Arredondo, D. L., Leyva-Gonzalez, M. A., Gonzalez-Morales, S. I., Lopez-Bucio, J., & Herrera-Estrella, L. (2014). Phosphate Nutrition: Improving Low-Phosphate Tolerance in Crops. In S. S. Merchant (Ed.), *Annual Review of Plant Biology, Vol 65* (Vol. 65, pp. 95-123). Palo Alto: Annual Reviews.
34. Mach, J. (2018). So Inclined: Phosphate Status and Leaf Angle in Rice. *30*(4), 743-744. doi:10.1105/tpc.18.00301 %J The Plant Cell
35. Maeda, E. (1965). Inhibition of lamina inclination by cytokinin in excised rice leaves. *Plant and Cell Physiology*, *6*(4), 653-660. doi:10.1093/oxfordjournals.pcp.a079137
36. Maeda, E. (1970). Geotropic Induction of the Lamina Inclination in Rice Leaves. *Japanese Journal of Crop Science*, *39*(2), 192-199. doi:10.1626/jcs.39.192
37. Meng, Z. B., Chen, L. Q., Suo, D., Li, G. X., Tang, C. X., & Zheng, S. J. (2012). Nitric oxide is the shared signalling molecule in phosphorus- and iron-deficiency-induced formation of cluster roots in white lupin (*Lupinus albus*). *Annals of Botany*, *109*(6), 1055-1064. doi:10.1093/aob/mcs024
38. Metusala, D. (2017). An alternative simple method for preparing and preserving cross-section of leaves and roots in herbaceous plants: Case study in Orchidaceae. *1862*(1), 030113. doi:10.1063/1.4991217
39. Mirazizi, F., Bahrami, A., Haghbeen, K., Shahbani Zahiri, H., Bakavoli, M., & Legge, R. L. (2016). Rapid and direct spectrophotometric method for kinetics studies and routine assay of peroxidase based on aniline diazo substrates. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *31*(6), 1162-1169. doi:10.3109/14756366.2015.1103234
40. Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*, *7*(9), 405-410. doi:10.1016/s1360-1385(02)02312-9
41. Moreno, M. A., Harper, L. C., Krueger, R. W., Dellaporta, S. L., & Freeling, M. (1997). *liguleless1* encodes a nuclear-localized protein required for induction of ligules and auricles during maize leaf organogenesis. *Genes & Development*, *11*(5), 616-628. doi:10.1101/gad.11.5.616
42. Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, *15*(3), 473-497. doi:10.1111/j.1399-3054.1962.tb08052.x
43. Neill, S. J., Desikan, R., Clarke, A., Hurst, R. D., & Hancock, J. T. (2002). Hydrogen peroxide and nitric oxide as signalling molecules in plants. *Journal of Experimental Botany*, *53*(372), 1237-1247. doi:10.1093/jexbot/53.372.1237 %J Journal of Experimental Botany
44. Niu, L., & Liao, W. (2016). Hydrogen peroxide signaling in plant development and abiotic responses: Crosstalk with nitric oxide and calcium. *Frontiers in Plant Science*, *7*(MAR2016). doi:10.3389/fpls.2016.00230
45. Papadopolou, E., & Grumet, R. (2005). Brassinosteroid-induced femaleness in cucumber and relationship to ethylene production. *HortScience*, *40*(6), 1763-1767. doi:10.21273/hortsci.40.6.1763
46. Phang, I. C., Leung, D. W. M., Taylor, H. H., & Burritt, D. J. (2011). The protective effect of sodium nitroprusside (SNP) treatment on Arabidopsis thaliana seedlings exposed to toxic level of Pb is not linked to avoidance of Pb uptake. *Ecotoxicology and Environmental Safety*, *74*(5), 1310-1315. doi:<https://doi.org/10.1016/j.ecoenv.2011.02.006>
47. Qiao, M., Sun, J., Liu, N., Sun, T., Liu, G., Han, S., . . . Wang, D. (2015). Changes of Nitric Oxide and Its Relationship with H₂O₂ and Ca²⁺ in Defense Interactions between Wheat and Puccinia Triticina. *PLoS One*, *10*(7), e0132265. doi:10.1371/journal.pone.0132265
48. Rather, B. A., Mir, I. R., Masood, A., Anjum, N. A., & Khan, N. A. (2020). Nitric Oxide Pre-Treatment Advances Seed Germination and Alleviates Copper-Induced Photosynthetic Inhibition in Indian Mustard. *Plants (Basel)*, *9*(6). doi:10.3390/plants9060776

49. Rozhon, W., Akter, S., Fernandez, A., & Poppenberger, B. (2019). Inhibitors of Brassinosteroid Biosynthesis and Signal Transduction. *Molecules (Basel, Switzerland)*, 24(23), 4372. doi:10.3390/molecules24234372
50. Ruan, W., Guo, M., Xu, L., Wang, X., Zhao, H., Wang, J., & Yi, K. (2018). An SPX-RLI1 Module Regulates Leaf Inclination in Response to Phosphate Availability in Rice. 30(4), 853-870. doi:10.1105/tpc.17.00738 %J The Plant Cell
51. Saito, S., Okamoto, M., Shinoda, S., Kushiro, T., Koshiba, T., Kamiya, Y., . . . Mizutani, M. (2006). A Plant Growth Retardant, Uniconazole, Is a Potent Inhibitor of ABA Catabolism in Arabidopsis. *Bioscience, biotechnology, and biochemistry*, 70, 1731-1739. doi:10.1271/bbb.60077
52. Schaller, G., & Binder, B. (2017). Inhibitors of Ethylene Biosynthesis and Signaling. In (Vol. 1573, pp. 223-235).
53. Schlagnhauer, C. D., & Arteca, R. N. (1985). Inhibition of brassinosteroid-induced epinasty in tomato plants by aminooxyacetic acid and Co²⁺. 65(2), 151-155. doi:<https://doi.org/10.1111/j.1399-3054.1985.tb02374.x>
54. Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany*, 2012, 217037. doi:10.1155/2012/217037
55. Shi, C., Qi, C., Ren, H., Huang, A., Hei, S., & She, X. (2015). Ethylene mediates brassinosteroid-induced stomatal closure via Gα protein-activated hydrogen peroxide and nitric oxide production in Arabidopsis. *Plant Journal*, 82(2), 280-301. doi:10.1111/tpj.12815
56. Shimada, A., Ueguchi-Tanaka, M., Sakamoto, T., Fujioka, S., Takatsuto, S., Yoshida, S., . . . Matsuoka, M. (2006). The rice SPINDLY gene functions as a negative regulator of gibberellin signaling by controlling the suppressive function of the DELLA protein, SLR1, and modulating brassinosteroid synthesis. *Plant Journal*, 48(3), 390-402. doi:10.1111/j.1365-3113X.2006.02875.x
57. Si, T., Wang, X., Wu, L., Zhao, C., Zhang, L., Huang, M., . . . Jiang, D. (2017). Nitric oxide and hydrogen peroxide mediate wounding-induced freezing tolerance through modifications in photosystem and antioxidant system in wheat. *Frontiers in Plant Science*, 8. doi:10.3389/fpls.2017.01284
58. Sies, H. (2017). Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox Biology*, 11, 613-619. doi:10.1016/j.redox.2016.12.035
59. Strader, L. C., Beisner, E. R., & Bartel, B. (2009). Silver ions increase auxin efflux independently of effects on ethylene response. *Plant Cell*, 21(11), 3585-3590. doi:10.1105/tpc.108.065185
60. Sylvester, A. W., Cande, W. Z., & Freeling, M. (1990). DIVISION AND DIFFERENTIATION DURING NORMAL AND LIGULELESS-1 MAIZE LEAF DEVELOPMENT. *Development*, 110(3), 985-1000.
61. Taulavuori, E., Hellström, E. K., Taulavuori, K., & Laine, K. (2001). Comparison of two methods used to analyse lipid peroxidation from *Vaccinium myrtillus* (L.) during snow removal, reacclimation and cold acclimation. *Journal of Experimental Botany*, 52(365), 2375-2380. doi:10.1093/jexbot/52.365.2375
62. Tewari, R. K., Kumar, P., Tewari, N., Srivastava, S., & Sharma, P. N. (2004). Macronutrient deficiencies and differential antioxidant responses - influence on the activity and expression of superoxide dismutase in maize. *Plant Science*, 166(3), 687-694. doi:10.1016/j.plantsci.2003.11.004
63. Tian, F., Bradbury, P. J., Brown, P. J., Hung, H., Sun, Q., Flint-Garcia, S., . . . Buckler, E. S. (2011). Genome-wide association study of leaf architecture in the maize nested association mapping population. *Nature Genetics*, 43(2), 159-U113. doi:10.1038/ng.746

64. Tollenaar, M., & Wu, J. (1999). Yield improvement in temperate maize is attributable to greater stress tolerance. *Crop Science*, 39(6), 1597-1604. doi:10.2135/cropsci1999.3961597x
65. Torres, G., Vossenkemper, J., Raun, W., & Taylor, R. (2011). MAIZE (ZEA MAYS) LEAF ANGLE AND EMERGENCE AS AFFECTED BY SEED ORIENTATION AT PLANTING. *Experimental Agriculture*, 47(4), 579-592. doi:10.1017/s001447971100038x
66. Trethewey, J. A. K., Rolston, M. P., McCloy, B. L., & Chynoweth, R. J. (2016). The plant growth regulator, trinexapac-ethyl, increases seed yield in annual ryegrass (*Lolium multiflorum* Lam.). *New Zealand Journal of Agricultural Research*, 59(2), 113-121. doi:10.1080/00288233.2015.1134590
67. Vardhini, B. V., & Anjum, N. A. (2015). Brassinosteroids make plant life easier under abiotic stresses mainly by modulating major components of antioxidant defense system. 2(67). doi:10.3389/fenvs.2014.00067
68. Veal, E., & Day, A. (2011). Hydrogen peroxide as a signaling molecule. *Antioxid Redox Signal*, 15(1), 147-151. doi:10.1089/ars.2011.3968
69. Velez-Ramirez, A. I., Van Ieperen, W., Vreugdenhil, D., & Millenaar, F. F. (2011). Plants under continuous light. *Trends in Plant Science*, 16(6), 310-318. doi:10.1016/j.tplants.2011.02.003
70. Wada, K., Marumo, S., Abe, H., Morishita, T., Nakamura, K., Uchiyama, M., & Mori, K. (1984). A rice lamina inclination test-A micro-quantitative bioassay for brassinosteroids. *Agricultural and Biological Chemistry*, 48(3), 719-726. doi:10.1080/00021369.1984.10866208
71. Wang, L., Guo, Y., Jia, L., Chu, H., Zhou, S., Chen, K., . . . Zhao, L. (2014). Hydrogen peroxide acts upstream of nitric oxide in the heat shock pathway in arabidopsis seedlings. *Plant Physiology*, 164(4), 2184-2196. doi:10.1104/pp.113.229369
72. Wang, L. H., Duan, G. L., Williams, P. N., & Zhu, Y. G. (2008). Influences of phosphorus starvation on OsACR2.1 expression and arsenic metabolism in rice seedlings. *Plant and Soil*, 313(1-2), 129-139. doi:10.1007/s11104-008-9685-2
73. Waskiewicz, A., Beszterda, M., & Goliński, P. (2014). Chapter 7. Nonenzymatic Antioxidants in Plants. In (pp. 201-234).
74. Xu, Y., Liu, F., Han, G., & Cheng, B. (2018). Genome-wide identification and comparative analysis of phosphate starvation-responsive transcription factors in maize and three other gramineous plants. *Plant Cell Rep*, 37(5), 711-726. doi:10.1007/s00299-018-2262-0
75. Yang, G., & Komatsu, S. (2000). Involvement of calcium-dependent protein kinase in rice (*Oryza sativa* L.) lamina inclination caused by brassinolide. *Plant and Cell Physiology*, 41(11), 1243-1250. doi:10.1093/pcp/pcd050
76. Yoshida, S., Navasero, S. A., & Ramirez, E. A. (1969). EFFECTS OF SILICA AND NITROGEN SUPPLY ON SOME LEAF CHARACTERS OF RICE PLANT. *Plant and Soil*, 31(1), 48-&. doi:10.1007/bf01373025
77. Yu, T. T., Liu, C. X., Lu, X. F., Bai, Y., Zhou, L., & Cai, Y. L. (2019). ZmAPRG, an uncharacterized gene, enhances acid phosphatase activity and Pi concentration in maize leaf during phosphate starvation. *Theoretical and Applied Genetics*, 132(4), 1035-1048. doi:10.1007/s00122-018-3257-5
78. ZHANG, S., WANG, S., XU, Y., YU, C., SHEN, C., QIAN, Q., . . . QI, Y. (2015). The auxin response factor, OsARF19, controls rice leaf angles through positively regulating OsGH3-5 and OsBRI1. 38(4), 638-654. doi:10.1111/pce.12397
79. Zhao, S. Q., Xiang, J. J., & Xue, H. W. (2013). Studies on the rice leaf inclination1 (LC1), an IAA-amido synthetase, reveal the effects of auxin in leaf inclination control. *Molecular Plant*, 6(1), 174-187. doi:10.1093/mp/sss064
80. Zhou, Y., Xu, X. Y., Chen, L. Q., Yang, J. L., & Zheng, S. J. (2012). Nitric oxide exacerbates Al-induced inhibition of root elongation in rice bean by affecting cell wall and plasma membrane properties. *Phytochemistry*, 76, 46-51. doi:<https://doi.org/10.1016/j.phytochem.2011.12.004>

5.1. Supplementary Figures

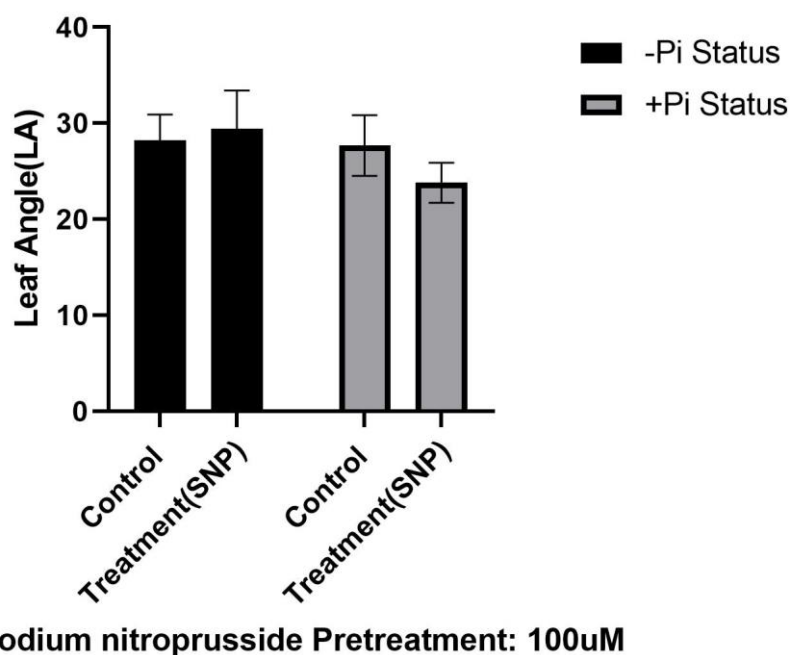


Figure S1. The effect of SNP seed pre-treatment on leaf inclination under differing phosphate status. Values indicated are mean leaf angles + SEM (n=5). Asterisks indicate the degree of significant difference.

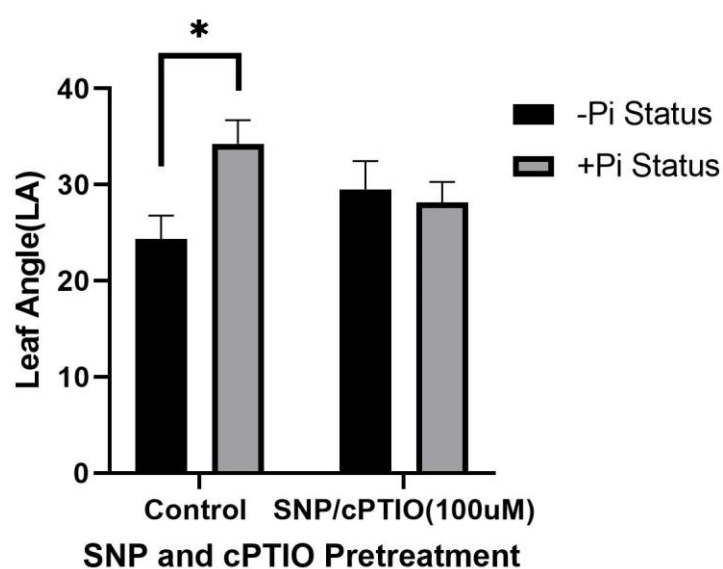


Figure S2. The effect of SNP(100uM) and cPTIO(100uM) seed pre-treatment on leaf inclination under differing phosphate status. Values indicated are mean leaf angles + SEM (n=5). Asterisks indicate the degree of significant difference.